



Final Report on Acute and Chronic Toxicity of Nitrate, Nitrite, Boron, Manganese, Fluoride, Chloride and Sulfate to Several Aquatic Animal Species

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Prepared for:

United States Environmental Protection Agency
Office of Science and Technology
Health and Ecological Criteria Division
and
U.S. EPA, Region 5 Water Division

EPA Contract: EP-C-09-001
Work Assignments: B-12 and 1-12
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FORWARD

The U.S. Environmental Protection Agency is charged under the Clean Water Act (§304(a)) with periodically updating water quality criteria (WQC) to protect existing and designated uses such as aquatic life and human health. Two States in EPA Region 5 identified the need to develop criteria for which no national guidance has been developed to date. To support these State efforts, EPA Region 5 contracted (Contract EP-C-09-001, Work Assignments B-12 and 1-12) with Great Lakes Environmental Center (GLEC) and its subcontractor, the Illinois Natural History Survey (INHS), to test the acute and chronic toxic effects of nitrate, nitrite, boron, manganese, fluoride, chloride and sulfate on several aquatic animal species. The results of this Work Assignment are contained in this report.

Any comments on this document should be sent to the Work Assignment Manager, Thomas Poleck at poleck.thomas@epa.gov or

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NOTICE: This document has been reviewed in accordance with U.S. EPA policy. While every effort was made to ensure the validity of the data in this report, any State, Tribe or other party should verify for themselves that the data meets all appropriate data quality requirements. For example, rearing and testing methods for *Hyalella* have not been standardized and therefore must be evaluated as to appropriateness in any dataset used for determining WQC. Mention of trade names or commercial products does not constitute endorsement or recommendation for use.

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EXECUTIVE SUMMARY

EPA guidance (EPA 1985) requires toxicity data from eight taxonomic groups, at a minimum, to derive a fully-supported water quality criterion. Two States in Region 5 are in the process of developing criteria for nitrate, boron, manganese and fluoride. This project was designed to fill existing data gaps by conducting toxicity testing on additional aquatic species for which data are absent from the literature. In addition, EPA Region 5 included several tests on nitrite, chloride and sulfate to meet other data needs and to support ongoing consultations with the U.S. Fish and Wildlife Service under the Endangered Species Act.

Specifically, the toxicity tests that were conducted are listed below along with their resultant lethal and effective concentrations.

A. Nitrate

1. *Lampsilis siliquoidea* (96-hour acute test)
[LC₅₀ = 357 mg/L]
2. *Megalonias nervosa* (96-hour acute test)
[LC₅₀ = 937 mg/L]
3. *Chironomus dilutus* (48-hour acute test)
[LC₅₀ = 278 mg/L]
4. *Amphinemura delosa* (96-hour acute test)
[LC₅₀ = 456 mg/L]
5. *Sphaerium simile* (96-hours acute test)
[LC₅₀ = 371 mg/L]
6. *Hyaella azteca* (96-hour acute test)
[LC₅₀ = 16.4 mg/L]
7. *Pimephales promelas* (96-hour acute test)
[LC₅₀ = 415 mg/L]
8. *Pimephales promelas* (32-day chronic test)
[Survival LC₅₀ = 76.8 mg/L, LC₂₅ = 68.2 mg/L, LC₂₀ = 64.6 mg/L,
LC₁₀ = 55.5 mg/L, NOEC = 49 mg/L, LOEC = 109 mg/L]
[Growth EC₅₀ = 91.3 mg/L, EC₂₅ = 65.3 mg/L, EC₂₀ = 59.8 mg/L,
EC₁₀ = 46.7 mg/L, NOEC = 49 mg/L, LOEC = 109 mg/L]

B. Nitrite

9. *Lampsilis siliquoidea* (96-hour acute test)
[LC₅₀ = 177 mg/L]
10. *Chironomus dilutus* (48-hour acute test)
[LC₅₀ = 15.6 mg/L]
11. *Amphinemura delosa* (96-hour acute test)
[LC₅₀ = 1.0 mg/L]
12. *Sphaerium simile* (96-hours acute test)
[LC₅₀ = 55.7 mg/L]
13. *Hyaella azteca* (96-hour acute test)
[LC₅₀ = 1.5 mg/L]

14. *Pimephales promelas* (96-hour acute test)
[LC₅₀ = 27.0 mg/L]
15. *Pimephales promelas* (32-day chronic test)
[Survival LC₅₀ = 11.3 mg/L, LC₂₅ = 7.0 mg/L, LC₂₀ = 5.7 mg/L,
LC₁₀ = 2.3 mg/L, NOEC = 6.8 mg/L, LOEC = 15.0 mg/L]
[Growth EC₅₀ = 14.6 mg/L, EC₂₅ = 11.7 mg/L, EC₂₀ = 11.0 mg/L,
EC₁₀ = 9.3 mg/L, NOEC = 6.8 mg/L, LOEC = 15.0 mg/L]
16. *Ceriodaphnia dubia* (48-hour acute test)
[LC₅₀ = 4.6 mg/L]
17. *Ceriodaphnia dubia* (7-day chronic test)
[Survival LC₅₀ = 4.7 mg/L, LC₂₅ = 3.5 mg/L, LC₂₀ = 3.3 mg/L,
LC₁₀ = 2.5 mg/L, NOEC = 3.6 mg/L, LOEC = 7.3 mg/L]
[Reproduction EC₅₀ = 2.4 mg/L, EC₂₅ = 1.9 mg/L, EC₂₀ = 1.7
mg/L, EC₁₀ = 1.4 mg/L, NOEC = 0.82 mg/L, LOEC = 1.8 mg/L]

C. Boron

18. *Lampsilis siliquioidea* (96-hour acute test)
[LC₅₀ = 137 mg/L]
19. *Megalonias nervosa* (96-hour acute test)
[LC₅₀ = >544 mg/L]
20. *Ligumia recta* (96-hour acute test)
[LC₅₀ = 147 mg/L]
21. *Pimephales promelas* (96-hour acute test)
[LC₅₀ = 101 mg/L]
22. *Pimephales promelas* (32-day chronic test)
[Survival LC₅₀ = 28.4 mg/L, LC₂₅ = 20.8 mg/L, LC₂₀ = 18.6 mg/L,
LC₁₀ = 12.8 mg/L, NOEC = 12.9 mg/L, LOEC = 27.4 mg/L]
[Growth EC₅₀ = 28.7 mg/L, EC₂₅ = 25.1 mg/L, EC₂₀ = 24.2 mg/L,
EC₁₀ = 21.9 mg/L, NOEC = <5.9 mg/L, LOEC = 5.9 mg/L]
23. *Ceriodaphnia dubia* (48-hour acute test at pH 7.75)
[LC₅₀ = 76.9 mg/L]
24. *Pimephales promelas* (96-hour acute test at pH 6.75)
[LC₅₀ = 70.6 mg/L]
25. *Pimephales promelas* (96-hour acute test at pH 7.75)
[LC₅₀ = 137 mg/L]
26. *Pimephales promelas* (96-hour acute test at pH 8.75)
[LC₅₀ = 133 mg/L]

D. Manganese

27. *Lampsilis siliquioidea* (96-hour acute test)
[LC₅₀ = 43.3 mg/L]
28. *Megalonias nervosa* (96-hour acute test)
[LC₅₀ = 31.5 mg/L]

E. Fluoride

29. *Sphaerium simile* (96-hours acute test)

[LC₅₀ = 62.0 mg/L]

30. *Hyaella azteca* (96-hour acute test)

[LC₅₀ = 13.4 mg/L]

F. Chloride

31. *Musculium transversum* (96-hours acute test)

[LC₅₀ = 1,930 mg/L]

G. Sulfate

32. *Ligumia recta* (96-hour acute test)

[LC₅₀ = 1,483 mg/L]

33. *Megalonaias nervosa* (96-hour acute test)

[LC₅₀ = 3,378 mg/L]

INTRODUCTION

There are no federal water quality criteria for the protection of aquatic life for nitrate, nitrite, boron, manganese and fluoride. States in Region 5 of the United States Environmental Protection Agency (EPA), particularly the states of Minnesota and Illinois, are in the process of revising or developing water quality criteria for each of these parameters, with the exception of nitrite. In addition, EPA recently completed a review of the available data and undertook a draft revision of the chloride criterion.

Nitrate and Nitrite

Biological field data generated by the State of Minnesota, as well as laboratory-derived toxicity test data, suggest that nitrate at concentrations similar to those observed in the surface waters of Minnesota may cause adverse effects to aquatic life. Much of the nitrate toxicity literature focuses on the effects of nitrate, especially the effects of wastes associated with fish cultures. More recent literature reports have documented that amphibians and invertebrates appear to be more sensitive to nitrate than fish.

The state of Minnesota is evaluating the need to develop a nitrate criterion to protect aquatic life as part of its current triennial Water Quality Standards (WQS) review. Efforts by Minnesota to develop aquatic life criteria for nitrate have involved a review of all the pertinent literature to date. The primary sources were the ECOTOX database, as well as a comprehensive search of the open literature. The scientific studies were each evaluated based on methodologies in the 1985 guidance of EPA (EPA 1985), and produced two observations: 1. there is a wide variation in response among aquatic organisms to nitrate toxicity; and 2. the research has documented the toxicity of nitrate for some aquatic organisms at environmentally relevant concentrations. In addition, the literature review found seven acceptable studies that reported acute toxicity values for eleven different species. Invertebrates (especially aquatic insects) were among the most sensitive taxa. Associated chronic values from these studies were found to be acceptable for three separate species, one each for a fish, an amphibian and a cladoceran. Unfortunately, these data are insufficient to develop Tier I aquatic life criteria for nitrate. Acceptable toxicity data are lacking for two required taxonomic categories: a benthic invertebrate and one other invertebrate.

Additional nitrite acute toxicity tests are also required to complete the data set for calculating a water quality criterion consistent with EPA's 1985 guidelines. Acute tests with an amphipod (e.g., *Hyaella azteca*) and a chironomid (e.g., *Chironomus dilutus*) would best serve this purpose. In addition, completing acute and chronic (early life stage) tests with the fathead minnow (*Pimephales promelas*) would augment existing data for fish, and add an additional acute to chronic ratio for calculating a chronic criterion.

Within Region 5, field data have shown elevated levels of nitrate. Given the relationship between nitrate and nitrite, the potential for nitrite to exist in the aquatic environment under certain circumstances, the toxic effects of nitrite, and the lack of nitrite data for

most organisms other than fish, EPA wanted to assess the potential need for a nitrite criterion by generating additional nitrite toxicity data for several species of organisms.

Boron, Manganese and Fluoride

The state of Illinois is in the process of revising its aquatic life criteria for boron, manganese and fluoride. The Illinois EPA conducted a literature review and identified the need for acute and chronic (early life stage) tests with *P. promelas* to add an additional acute to chronic ratio for calculating a chronic criterion for boron. Additionally, recent toxicity data suggest that pH affects the toxicity of boron, which is consistent with the fact that borate exists in water as a pH-dependent mixture of various borate ions. Therefore, EPA wanted to assess the effects of pH on the toxicity of boron on *Ceriodaphnia dubia* and *P. promelas*.

For fluoride, the data needs include acute and chronic tests on *Hyalella*, in order to develop an additional acute to chronic ratio with an organism other than *Pimephales* or *Daphnia*. The available acute data for an amphipod also need to be supplemented with tests conducted using a range of test water hardness levels. In order to facilitate the review of these criteria, and to provide additional support for the database, EPA also wanted to generate freshwater mussel toxicity data.

Chloride

EPA recently reviewed and developed additional toxicity data for chloride. As part of the review of that data and subsequent development of a draft revision of the criteria, EPA determined that additional toxicity information on resident fingernail clams (other than those from the genus *Sphaerium*) is desirable. To facilitate the acquisition of these data, EPA is interested in additional toxicity tests on other resident fingernail clams, such as *Musculium sp.*

Sulfate

Additional sulfate toxicity data are required to enhance EPA's basis for making a determination under the Endangered Species Act (ESA) that the recently adopted sulfate criteria by Illinois and Indiana are not likely to adversely affect threatened and endangered species. EPA Region 5 is currently consulting under Section 7 of the ESA on the Agency's approval of the Illinois and Indiana surface water quality standards. Data on the sensitivity of mussels are limited and mussels are often one of the most sensitive species to adverse water quality conditions. Therefore, EPA wanted to generate sulfate toxicity data for two mussel species (*Ligumia recta* and *Megaloniais nervosa*).

Because of the data needs outlined above, Great Lakes Environmental Center (GLEC) and its subcontractor the Illinois Natural History Survey (INHS) were contracted by the EPA to augment existing toxicity data through a series of toxicity tests. This document presents in detail the materials and methods by which GLEC and INHS performed these

toxicity tests with nitrate, nitrite, boron, manganese, fluoride, sulfate and chloride. Also included are the results of the toxicity tests, including % survival and mortality, growth and reproduction (where appropriate), water chemistry data, statistical analyses performed on the data and toxicology endpoint estimates.

MATERIALS AND METHODS

GLEC and INHS performed a total of 33 acute and chronic toxicity tests (Table 1). Tests on *C. dubia*, *C. dilutus*, *H. azteca* and *P. promelas* were completed by GLEC; tests on all other species were completed by INHS. Eight of these tests were designed to determine acute and/or chronic toxicity of the chemical nitrate to seven different species of aquatic organisms; nine additional tests were designed to determine acute and/or chronic toxicity of the chemical nitrite to seven different species of aquatic organisms. Five different species of aquatic organisms were exposed to boron under acute and/or chronic conditions during nine separate toxicity tests while two aquatic organisms were exposed to manganese under acute conditions. Acute toxicity was determined for fluoride, chloride and sulfate using one of four species of mussels and *H. azteca*.

Toxicity testing under this Work Assignment was accomplished using the methods outlined in the ASTM standards (Table 2), GLEC and INHS standard operating procedures (GLEC 2009 a, GLEC 2009 b, GLEC 2009 c, GLEC 2009 d, GLEC 2009 e, GLEC 2009 f, GLEC 2009 g and INHS 2009) and the Quality Assurance Project Plan (GLEC 2009) developed for this Work Assignment. Prior to completing the toxicity test for each test chemical/organism combination, range-finding toxicity tests were initiated to determine the appropriate test chemical concentrations that would “bracket” the LC₅₀ estimates. The range of concentrations for these tests was determined either from the literature or from laboratory experiences with similar organisms. Once the appropriate range in test chemical concentrations was defined for each test chemical/organism combination, definitive toxicity testing was completed.

Dilution water used for the tests consisted of either EPA Moderately Hard Reconstituted Water (MHRW) (EPA 2002a and EPA 2002 b), EPA Dilute Mineral Water (DMW) (EPA 2002 a and EPA 2002 b) or de-chlorinated Lake Michigan water (City of Traverse City, Michigan water passed through an activated carbon filter). The chemical properties of the de-chlorinated Lake Michigan water are presented in Table 3; these data were generated both before and after the *P. promelas* flow-through test with boron at pH 8.75.

Water quality parameters (water temperature, pH, dissolved oxygen (DO), conductivity, hardness, alkalinity and ammonia) were measured throughout the tests at pre-determined, test-specific intervals. Water temperature, pH, DO and conductivity were measured *in situ* using calibrated meters. Hardness, alkalinity and ammonia were measured in water collected from test chambers using methods outlined in SM 2340 C (APHA *et al.* 2006 a), SM 2320 B (APHA *et al.* 2006 b) and SM 4500-NH₃ (APHA *et al.* 2006 c), respectively. Test concentrations of the target test chemicals were also verified using the analytical chemistry methods outlined in Table 4 and laboratory-specific standard operating procedures (GLEC 2008, GLEC 2009 h, Simmerman 2008, UL 2008 a, UL 2008 b, UL 2009 a and UL 2009 b). For toxicity testing completed by GLEC, target test chemicals were verified by the GLEC analytical chemistry laboratory (nitrate and nitrite) or SOS Analytical (boron and fluoride). Target test chemical sample analysis for toxicity testing completed by INHS was completed by the INHS analytical laboratory (nitrate and nitrite) or Underwriters Laboratories (boron, fluoride, chloride, sulfate and manganese).

Once the toxicity tests were complete, statistical analysis of the mortality (and where appropriate in chronic tests, growth or reproduction) data was completed following guidance outlined in EPA (2002 a), EPA (2002 b) and the ASTM methods (Table 2). Specifically, data were analyzed using either TOXSTAT software (Gulley 1996) or EPA's Toxicity Relationship Analysis Program (TRAP, Version 1.00). Where appropriate, data were transformed to meet the assumptions of normality and homogeneity of variance. Analysis of variance (ANOVA), parametric (e.g. Probit) or non-parametric (e.g. Spearman-Kärber) tests and mathematical models (TRAP) were then used to determine appropriate toxicology endpoints, including: LC_{50} , LC_{50} , LC_{25} , LC_{20} and LC_{10} estimates; EC_{50} , EC_{25} , EC_{20} , EC_{10} estimates for growth or reproduction; and No Observed Effects Concentration (NOEC) and Lowest Observed Effects Concentration (LOEC) estimates.

Table 1. Experimental matrix for toxicity testing completed by GLEC and INHS.

Test Species and Duration	Nitrate	Nitrite	Boron	Manganese
<i>Ceriodaphnia dubia</i> - 48 hr		X		
<i>Lampsilis siliquioidea</i> 96 hr	X	X	X	X
<i>Chironomus dilutus</i> - 48 hr	X	X		
<i>Amphinemura delosa</i> - 96 hr	X	X		
<i>Sphaerium simile</i> - 96 hr	X	X		
<i>Hyaella azteca</i> - 96 hr	X	X		
<i>Pimephales promelas</i> - 96 hr	X	X	X	
<i>Pimephales promelas</i> - 32 day	X	X	X	
<i>Ceriodaphnia dubia</i> - 7 day		X		
<i>Ceriodaphnia dubia</i> - 48 hr (pH 7.75)			X	
<i>Pimephales promelas</i> - 96 hr (pH 6.75)			X	
<i>Pimephales promelas</i> - 96 hr (pH 7.75)			X	
<i>Pimephales promelas</i> - 96 hr (pH 8.75)			X	
<i>Ligumia recta</i> - 96 hr			X	
<i>Megaloniaias nervosa</i> - 96 hr	X		X	X

Test Species and Duration	Fluoride	Chloride	Sulfate
<i>Sphaerium simile</i> - 96 hr	X		
<i>Hyaella azteca</i> - 96 hr	X		
<i>Musculium transversum</i> - 96 hr		X	
<i>Ligumia recta</i> - 96 hr			X
<i>Megaloniaias nervosa</i> - 96 hr			X

Table 2. ASTM standard used for each toxicity test completed by GLEC and INHS.

Test Species and Duration	Nitrate	Nitrite	Boron
<i>Ceriodaphnia dubia</i> - 48 hr		ASTM E729	
<i>Lampsilis siliquioidea</i> - 96 hr	ASTM 2455-06	ASTM 2455-06	ASTM 2455-06
<i>Chironomus dilutus</i> - 48 hr	ASTM E729	ASTM E729	
<i>Amphinemura delosa</i> - 96 hr	ASTM E729	ASTM E729	
<i>Sphaerium simile</i> - 96 hr	ASTM E729	ASTM E729	
<i>Hyalella azteca</i> - 96 hr	ASTM E729	ASTM E729	
<i>Pimephales promelas</i> - 96 hr	ASTM E729	ASTM E729	ASTM E729
<i>Pimephales promelas</i> - 32 day	ASTM E1241	ASTM E1241	ASTM E1241
<i>Ceriodaphnia dubia</i> - 7 day		ASTM E1295	
<i>Ceriodaphnia dubia</i> - 48 hr (pH 7.75)			ASTM E729
<i>Pimephales promelas</i> - 96 hr (pH 6.75)			ASTM E729
<i>Pimephales promelas</i> - 96 hr (pH 7.75)			ASTM E729
<i>Pimephales promelas</i> - 96 hr (pH 8.75)			ASTM E729
<i>Ligumia recta</i> - 96 hr			ASTM 2455-06
<i>Megaloniaias nervosa</i> - 96 hr	ASTM 2455-06		ASTM 2455-06

Test Species and Duration	Manganese	Fluoride	Chloride
<i>Sphaerium simile</i> - 96 hr		ASTM E729	
<i>Lampsilis siliquioidea</i> - 96 hr	ASTM 2455-06		
<i>Hyalella azteca</i> - 96 hr		ASTM E729	
<i>Musculium transversum</i> - 96 hr			ASTM E729
<i>Megaloniaias nervosa</i> - 96 hr	ASTM 2455-06		

Test Species and Duration	Sulfate
<i>Ligumia recta</i> - 96 hr	ASTM 2455-06
<i>Megaloniaias nervosa</i> - 96 hr	ASTM 2455-06

Table 3. Chemical properties of de-chlorinated Lake Michigan (city of Traverse City) water.

Parameter	Level of Detection (mg/L)	2/12/2010 Result (mg/L)	2/16/2010 Result (mg/L)
Total Aluminum	0.05	ND	0.05
Dissolved Aluminum	0.05	ND	ND
Total Antimony	0.002	ND	ND
Total Arsenic	0.002	0.003	ND
Total Barium	0.05	ND	ND
Total Beryllium	0.001	ND	ND
Total Cadmium	0.001	ND	ND
Total Calcium	0.1	42.6	32.8
Chloride *	1	14	
Total Chromium	0.005	ND	ND
Total Cobalt	0.02	ND	ND
Total Copper	0.004	ND	ND
Total Iron	0.05	ND	ND
Total Lead	0.002	ND	ND
Total Magnesium	5.0	13.7	12.3
Total Manganese	0.05	ND	ND
Total Mercury	0.0005	ND	ND
Total Nickel	0.05	ND	ND
Total Potassium	0.1	1.42	1.33
Total Selenium	0.005	0.006	ND
Total Silver	0.001	ND	ND
Total Sodium	0.5	8.83	7.75
Sulfate *	2	29	
Total Thallium	0.002	ND	ND
Total Vanadium	0.004	ND	ND
Total Zinc	0.05	ND	ND

ND = Below level of detection.

* Sample collected on November 11, 2010.

Table 4. Analytical chemistry methods for each toxicity test completed by GLEC and INHS.

Test Species and Duration	Nitrate	Nitrite
<i>Ceriodaphnia dubia</i> - 48 hr		SM 4500 NO ₃ and SM 4500 NO ₂
<i>Lampsilis siliquoidea</i> - 96 hr	EPA 353.1 and EPA 354.1	EPA 353.1 and EPA 354.1
<i>Chironomus dilutus</i> - 48 hr	SM 4500 NO ₃ and SM 4500 NO ₂	SM 4500 NO ₃ and SM 4500 NO ₂
<i>Amphinemura delosa</i> - 96 hr	EPA 353.1 and EPA 354.1	EPA 353.1 and EPA 354.1
<i>Sphaerium simile</i> - 96 hr	EPA 353.1 and EPA 354.1	EPA 353.1 and EPA 354.1
<i>Hyalella azteca</i> - 96 hr	SM 4500 NO ₃ and SM 4500 NO ₂	SM 4500 NO ₃ and SM 4500 NO ₂
<i>Pimephales promelas</i> - 96 hr	SM 4500 NO ₃ and SM 4500 NO ₂	SM 4500 NO ₃ and SM 4500 NO ₂
<i>Pimephales promelas</i> - 32 day	SM 4500 NO ₃ and SM 4500 NO ₂	SM 4500 NO ₃ and SM 4500 NO ₂
<i>Ceriodaphnia dubia</i> - 7 day		SM 4500 NO ₃ and SM 4500 NO ₂
<i>Megaloniais nervosa</i> - 96 hr	EPA 353.1 and EPA 354.1	

Test Species and Duration	Boron	Manganese
<i>Ceriodaphnia dubia</i> - 48 hr		
<i>Lampsilis siliquoidea</i> - 96 hr	ICPMS 200.8	ICPMS 200.7
<i>Pimephales promelas</i> - 96 hr	EPA 200.8	
<i>Pimephales promelas</i> - 32 day	EPA 200.8	
<i>Ceriodaphnia dubia</i> - 48 hr (pH 7.75)	EPA 200.8	
<i>Pimephales promelas</i> - 96 hr (pH 6.75)	EPA 200.8	
<i>Pimephales promelas</i> - 96 hr (pH 7.75)	EPA 200.8	
<i>Pimephales promelas</i> - 96 hr (pH 8.75)	EPA 200.8	
<i>Ligumia recta</i> - 96 hr	ICPMS 200.8	
<i>Megaloniais nervosa</i> - 96 hr	ICPMS 200.8	ICPMS 200.7

Test Species and Duration	Fluoride	Chloride
<i>Sphaerium simile</i> - 96 hr	EPA 300.0	
<i>Hyalella azteca</i> - 96 hr	EPA 300.0	
<i>Musculium transversum</i> - 96 hr		EPA 300.0

Test Species and Duration	Sulfate
<i>Ligumia recta</i> - 96 hr	EPA 300.0
<i>Megaloniais nervosa</i> - 96 hr	EPA 300.0

CHEMICALS TESTED AND TOXICITY TESTS PERFORMED

Nitrate

Table 5 provides a summary of estimated LC₅₀ values for the eight toxicity tests performed using nitrate. LC₅₀ values ranged between 16.4 and 937 mg NO₃-N/L.

Table 5. LC₅₀ estimates for toxicity tests performed using nitrate.

Test Species and Duration	LC ₅₀ (mg NO ₃ -N/L)
<i>Lampsilis siliquoidea</i> - 96 hr	357
<i>Chironomus dilutus</i> - 48 hr	278
<i>Amphinemura delosa</i> - 96 hr	456
<i>Sphaerium simile</i> - 96 hr	371
<i>Hyalella azteca</i> - 96 hr	16.4
<i>Pimephales promelas</i> - 96 hr	415
<i>Pimephales promelas</i> - 32 day	76.8
<i>Megaloniaias nervosa</i> - 96 hr	937

For each of the acute toxicity tests completed using nitrate, two tables were generated: the first summarizes the test results for each toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity tests. The results of chronic tests performed with nitrate were summarized in three tables: the first summarizes nominal and analytical test concentrations, LC₅₀ estimates with confidence intervals, NOEC and LOEC estimates, mean survival and mean biomass; the second table summarizes replicate-specific survival and growth data and the third table summarizes analytical chemistry data collected throughout the toxicity tests. Also discussed, if applicable, are deviations from the guidance provided in the ASTM method used to complete the toxicity testing.

Nitrate and nitrite concentration data generated by the GLEC analytical chemistry laboratory were reported as nitrate (mg NO₃/L) and nitrite (mg NO₂/L). For consistency (i.e. so that the data correspond to the nitrate and nitrogen data generated by the analytical chemistry laboratory used by the INHS), the GLEC data were converted to nitrate-nitrogen (N-NO₃/L) and nitrite-nitrogen (N-NO₂/L) for LC₅₀ determinations and reporting purposes. The data as reported by the GLEC analytical laboratory are available in the appendices.

96-hr Toxicity of Nitrate on *Lampsilis siliquoidea*

The 96-hr test to determine the toxicity of nitrate on *L. siliquoidea* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Missouri State University laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 1,129, 564, 282, 141, and 70 mg N-NO₃/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 6; test results are provided in Table 7. Analytical chemistry data are provided in Table 8. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 1.

Table 6. Test conditions for 96-hour toxicity test on *Lampsilis siliquoidea* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Lampsilis siliquoidea</i> , juveniles <5 days old, Missouri State University
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	June 03 - 07, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg N-NO ₃ /L):	1,129, 564, 282, 141, and 70
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg N-NO ₃ /L):	1,144, 580, 291, 143, and 74
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent, Cas. No. 7631-99-4, Lot # B0110978
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 7. Test results for 96-hour toxicity test on *Lampsilis siliquoidea* with nitrate.

Results of a <i>Lampsilis siliquoidea</i> 96-Hour Static Acute Toxicity Test								
Conducted <u>06/03/09 - 06/07/09</u> Using: Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	5	10	10	10	815	598	491	357
70 (74) mg/L	0	0	15	25	96-Hour LC ₅₀ * = 357 mg/L			
141 (143) mg/L	0	5	5	25				
					LC ₅₀ 95% Confidence Limits			
282 (291) mg/L	5	10	20	35	24-Hr	48-Hr	72-Hr	96-Hr
564 (580) mg/L	15	30	55	75	LL 730	498	383	250
					UL 909	717	631	509
1129 (1144) mg/L	85	100	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 8. Analytical chemistry data for 96-hour toxicity test on *Lampsilis siliquoidea* with nitrate.

Nominal (Measured) Test Concentration		N-NO ₂ ^a (mg/L)	N-NO ₃ ^{a,b} (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	<0.01	<0.01	20.1	8.0	7.81	305	60	90
	Day 1			19.9					
	Day 2			20.0					
	Day 3			20.1					
	Day 4	<0.01	<0.01	19.9	8.0	7.77	310	62	92
			na						
70 (74) mg/L	Day 0	<0.01	73	20.1	7.9	8.01	912	60	90
	Day 1			19.9					
	Day 2			20.0					
	Day 3			20.0					
	Day 4	<0.01	75	19.9	8.0	7.82	920	62	92
			74						
141 (143) mg/L	Day 0	<0.01	144	20.1	7.9	8.00	1484	60	90
	Day 1			19.8					
	Day 2			20.1					
	Day 3			20.1					
	Day 4	<0.01	142	19.9	8.0	7.85	1500	62	92
			148						
282 (291) mg/L	Day 0	<0.01	283	20.0	7.9	8.05	2610	60	90
	Day 1			19.8					
	Day 2			20.0					
	Day 3			20.1					
	Day 4	<0.01	299	19.9	8.0	7.72	2620	62	92
			291						
564 (580) mg/L	Day 0	<0.01	582	20.0	7.9	8.10	4760	60	90
	Day 1			19.8					
	Day 2			20.0					
	Day 3			20.0					
	Day 4	<0.01	579	20.0	7.9	7.86	4770	62	92
			580						
1129 (1144) mg/L	Day 0	<0.01	1158	20.0	7.9	8.12	8720	60	90
	Day 1			19.9					
	Day 2			20.0					
	Day 3			20.1					
	Day 4	<0.01	1131	19.9	7.9	7.81	8720	62	92
			1144						

^a Nitrite Analysis Method 354.1

^b Nitrite + Nitrate Analysis Method 353.1

na = not applicable

96-hr Toxicity of Nitrate on *Megalonaias nervosa*

The 96-hr test to determine the toxicity of nitrate on *M. nervosa* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Genoa National Fish Hatchery culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate. In one replicate of the 282 mg/L treatment, a test organism was inadvertently crushed, but this was accounted for in the LC₅₀ calculation.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 2,258, 1,129, 564, 282, and 141 mg N-NO₃/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 9; test results are provided in Table 10. Analytical chemistry data are provided in Table 11. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 2.

Table 9. Test conditions for 96-hour toxicity test on *Megaloniaias nervosa* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Megaloniaias nervosa</i> , juveniles <5 days old (Genoa National Fish Hatchery)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	October 23 - 27, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg N-NO ₃ /L):	2,258, 1,129, 564, 282, and 141
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- N-NO ₃ /L):	2,002, 1,108, 533, 279, and 138
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent, Cas. No. 7631-99-4, Lot # B0110978
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 10. Test results for 96-hour toxicity test on *Megalonaias nervosa* with nitrate.

Results of a <i>Megalonaias nervosa</i> 96-Hour Static Acute Toxicity Test								
Conducted <u>06/03/09 - 06/07/09</u> Using: Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	5	5	5	5	1,203	1,035	969	937
141 (138) mg/L	0	0	0	0	96-Hour LC ₅₀ * = 937 mg/L			
282 (279) mg/L	0	0	0	0				
					LC ₅₀ 95% Confidence Limits			
564 (533) mg/L	0	0	0	0	24-Hr	48-Hr	72-Hr	96-Hr
1129 (1108) mg/L	40	55	65	70	LL 991	893	841	818
					UL 1,460	1,199	1,116	1,073
2258 (2002) mg/L	90	100	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 11. Analytical chemistry data for 96-hour toxicity test on *Megalonaias nervosa* with nitrate.

Nominal (Measured) Test Concentration		N-NO ₂ ^a	N-NO ₃ ^{a,b}	Temp.	pH	D.O.	Cond.	Alkalinity	Hardness	Ammonia
		(mg/L)	(mg/L)	(°C)	(s.u.)	(mg/L)	(mmos)	(mg/L)	(mg/L)	(mg/L)
Dilution water/Control	Day 0	<0.02	<0.07	20.9	8.0	8.34	303	60	90	<0.05
	Day 1			20.9	8.0	8.13	304			
	Day 2			20.8	8.1	8.26	306			
	Day 3			20.9	8.0	8.36	305			
	Day 4	<0.02	<0.07	20.8	8.0	8.08	315	60	92	<0.05
			na							
141 (138) mg/L	Day 0	<0.02	132.6	20.8	8.2	8.34	1454	60	90	<0.05
	Day 1			20.9	8.1	8.06	1421			
	Day 2			20.8	8.1	8.34	1383			
	Day 3			20.9	7.8	8.34	1370			
	Day 4	<0.02	143.4	20.9	8.0	7.92	1361	60	90	<0.05
			138							
282 (279) mg/L	Day 0	<0.02	275.6	20.8	8.2	8.35	2550	60	90	<0.05
	Day 1			20.9	8.1	8.15	2520			
	Day 2			20.8	8.1	8.25	2470			
	Day 3			20.9	7.9	8.34	2480			
	Day 4	<0.02	283.4	20.9	8.0	7.95	2480	62	90	<0.05
			279							
564 (533) mg/L	Day 0	<0.02	543.3	20.9	8.2	8.35	4680	60	90	<0.05
	Day 1			20.9	8.1	8.08	4610			
	Day 2			20.8	8.1	8.16	4560			
	Day 3			20.9	7.9	8.32	4560			
	Day 4	<0.02	523.7	20.9	8.0	7.92	4610	62	92	<0.05
			533							
112 (1108) mg/L	Day 0	<0.02	1189.4	20.9	8.1	8.39	8710	60	90	<0.05
	Day 1			20.9	8.1	8.15	8650			
	Day 2			20.8	8.0	8.24	8530			
	Day 3			20.9	8.0	8.30	8440			
	Day 4	<0.02	1032.8	20.9	8.0	7.96	8540	62	92	<0.05
			1108							
2258 (2002) mg/L	Day 0	<0.02	2011.8	20.9	8.1	8.43	16240	60	90	<0.05
	Day 1			20.9	8.1	8.16	16000			
	Day 2			20.8	8.0	8.16	15690			
	Day 3			20.9	8.0	8.25	15810			
	Day 4	<0.02	1992.2	20.9	8.0	7.90	15910	64	92	<0.05
			2002							

^aNitrite Analysis Method 354.1

^bNitrite + Nitrate Analysis Method 353.1

na = not applicable

temp. = temperature; Cond. = conductivity

48-hr Toxicity of Nitrate on *Chironomus dilutus*

The 48-hr test to determine the toxicity of nitrate on *C. dilutus* was completed by GLEC. Test organisms, 10-days old collected from the GLEC laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 88, 146, 244, 406, and 678 mg N-NO₃/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 12; test results are provided in Table 13. Analytical chemistry data are provided in Table 14. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 3.

Table 12. Test conditions for 48-hour toxicity test on *Chironomus dilutus* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Chironomus dilutus</i> , 10 days old, GLEC culture
2. Test Type and Duration:	Static, 48 hours
3. Test Dates:	July 23-July 25, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	100 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg N-NO ₃ /L):	678, 406, 244, 146, and 88
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg N-NO ₃ /L):	621, 372, 255, 166, and 91
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent Cas. No. 7631-99-4, Batch # 66696LJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 13. Test results for 48-hour toxicity test on *Chironomus dilutus* with nitrate.

Results of a <i>Chironomus dilutus</i> (tentans) 48-Hour Static Acute Toxicity Test								
Conducted 07/23/09 - 07/25/09 Using: Nitrate (Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ * Values (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)			>621	278	NA	NA
88 (91) mg/L	0 (0)	0 (0)			48-Hour LC ₅₀ * = 278 mg/L			
146 (166) mg/L	0 (0)	15 (15)			LC ₅₀ * 95% Confidence Limits			
244 (255) mg/L	15 (15)	45 (45)			24-Hr	48-Hr	72-Hr	96-Hr
407 (372) mg/L	20 (20)	65 (65)			LL NA	239	NA	NA
					UL NA	325	NA	NA
678 (621) mg/L	25 (25)	100 (100)			LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on the measured concentrations.

Table 14. Analytical chemistry data for 48-hour toxicity test on *Chironomus dilutus* with nitrate.

Nominal (Measured) Test Concentration		Nitrate ^b (mg NO3-N/L)	Nitrite ^a (mg NO2-N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.0012	<0.0011	22.3	7.98	7.4	292	60	84	ND
		<0.0012								
	Day 1			21.3	7.94	7.7				
	Day 2	<0.0012	0.0084	22.4	7.84	7.6	290	90	124	
		0.0								
88 (91) mg/L	Day 0	83.6	<0.0011	22.1	7.99	7.5	854			
	Day 1			21.3	7.90	7.7				
	Day 2	98.3	0.0150	22.4	7.87	7.7	860			
		90.9								
146 (166) mg/L	Day 0	187.9	<0.0011	22.1	7.98	7.6	1368			
	Day 1			21.3	7.90	7.7				
	Day 2	143.7	0.0072	22.3	7.89	7.5	1304			
		165.8								
244 (255) mg/L	Day 0	255.3	0.0016	22.6	7.95	7.6	2030			
			0.0013							
	Day 1			21.4	7.86	7.7				
	Day 2	255.9	0.0110	22.3	7.94	7.5	2000			
		255.6								
407 (372) mg/L	Day 0	325.3	0.0024	22.7	7.96	7.7	2860			
	Day 1			21.4	7.85	7.8				
	Day 2	419.0	0.0112	22.3	7.93	7.7	2740			
		372.1								
678 (621) mg/L	Day 0	607.6	0.0025	22.2	7.95	7.9	4900	60	120	ND
	Day 1			21.4	7.8	8.0				
	Day 2	634.7	0.0173	22.3	7.93	7.5	4870	64	136	
		621.2								

^a Nitrite Analysis Method SM 4500 NO2

^b Nitrate Analysis Method SM 4500 NO3

ND Not Detect; below detection limit

96-hr Toxicity of Nitrate on *Amphinemura delosa*

The 96-hr test to determine the toxicity of nitrate on *A. delosa* was completed by INHS. Test organisms, field collected nymphs, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with four organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 2,258, 1,129, 564, 282, and 141 mg N-NO₃/L.

Testing was conducted at 12 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 15; test results are provided in Table 16. Analytical chemistry data are provided in Table 17. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 4.

Table 15. Test conditions for 96-hour toxicity test on *Amphinemura delosa* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Amphinemura delosa</i> , field-collected nymphs
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	May 14 - 18, 2009
4. Test Temperature (°C):	12 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	250 mL beaker
9. Volume of Test Solutions:	200 mL
10. No. of Test Organisms per Test Vessel:	4
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	16
13. Test Concentrations (mg N-NO ₃ /L):	2,258, 1,129, 564, 282, and 141
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg N-NO ₃ /L):	2,286, 1,230, 596, 274, and 152
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USPEA MHRW
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent, Cas. No. 7631-99-4, Lot # B0110978
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 16. Test results for 96-hour toxicity test on *Amphinemura delosa* with nitrate.

Results of a <u><i>Amphinemura delosa</i></u>		<u>96-Hour Static Acute Toxicity Test</u>						
Conducted <u>05/14/09 - 05/18/09</u>		<u>Using: Nitrate (Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4)</u>						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	1753	584	499	456
141 (152) mg/L	12.5	12.5	12.5	12.5	96-Hour LC ₅₀ * = 456 mg/L			
282 (274) mg/L	6.25	31.25	37.5	37.5				
					LC ₅₀ 95% Confidence Limits			
564 (596) mg/L	6.25	37.5	43.75	50	24-Hr	48-Hr	72-Hr	96-Hr
1129 (1230) mg/L	25	81.25	87.5	93.75	LL 1394	409	352	325
					UL 2204	834	706	642
2258 (2286) mg/L	68.75	100	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC50 Confidence Limit Values: Spearman-Karber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 17. Analytical chemistry data for 96-hour toxicity test on *Amphinemura delosa* with nitrate.

Nominal (Measured) Test Concentration		N-NO ₂ ^a	N-NO ₃ ^{a,b}	Temperature	pH	D.O.	Conductivity	Alkalinity	Hardness
		(mg/L)	(mg/L)	(°C)	(s.u.)	(mg/L)	(µmhos)	(mg/L)	(mg/L)
Dilution water/Control	Day 0	<0.08	<0.08	12.5	7.9	8.80	298	60	90
	Day 1			12.3	7.9		297		
	Day 2			12.4	7.9		297		
	Day 3			12.5	7.8		300		
	Day 4	<0.07	<0.07	12.8	7.8	9.85	298	60	88
			na						
141 (152) mg/L	Day 0	<0.08	161	12.5	8.0	8.80	1440	60	92
	Day 1			12.4	7.9		1455		
	Day 2			12.4	7.9		1458		
	Day 3			12.5	7.9		1460		
	Day 4	<0.08	143	12.8	7.9	9.57	1441	60	92
			152						
282 (274) mg/L	Day 0	<0.08	274	12.5	8.0	9.09	2540	60	92
	Day 1			12.3	7.9		2560		
	Day 2			12.4	7.9		2560		
	Day 3			12.5	7.9		2560		
	Day 4	<0.07	274	12.8	7.9	9.92	2540	62	92
			274						
564 (596) mg/L	Day 0	<0.08	599	12.5	8.0	9.09	4690	60	92
	Day 1			11.9	7.9		4690		
	Day 2			12.4	7.9		4700		
	Day 3			12.5	7.9		4710		
	Day 4	<0.07	593	12.8	7.9	9.93	4650	60	92
			596						
1129 (1230) mg/L	Day 0	<0.08	1260	12.5	8.0	9.11	8720	60	92
	Day 1			11.9	7.9		8770		
	Day 2			12.4	7.9		8780		
	Day 3			12.5	7.9		8790		
	Day 4	<0.07	1200	12.8	7.9	9.92	8670	62	92
			1230						
2258 (2286) mg/L	Day 0	<0.08	2200	12.5	7.9	9.08	16260	60	92
	Day 1			12.0	7.9		16270		
	Day 2			12.4	7.9		16270		
	Day 3			12.5	7.9		16280		
	Day 4	<0.07	2375	12.8	7.9	9.97	16230	60	92
			2286						

^a Nitrite Analysis Method 354.1

^b Nitrite + Nitrate Analysis Method 353.1

na = not applicable

96-hr Toxicity of Nitrate on *Sphaerium simile*

The 96-hr test to determine the toxicity of nitrate on *S. simile* was completed by INHS. Test organisms, juveniles released from field-collected adults, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 1,129, 564, 282, 141, and 71 mg N-NO₃/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 18; test results are provided in Table 19. Analytical chemistry data are provided in Table 20. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 5.

Table 18. Test conditions for 96-hour toxicity test on *Sphaerium simile* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Sphaerium simile</i> , juveniles (released from field-collected adults)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	July 21- 25, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	120 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg N-NO ₃ /L):	1,129, 564, 282, 141, and 71
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg N-NO ₃ /L):	1,125, 569, 272, 132, and 65
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USPEA MHRW
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent, Cas. No. 7631-99-4, Lot # B0110978
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 19. Test results for 96-hour toxicity test on *Sphaerium simile* with nitrate.

Results of a <i>Sphaerium simile</i>		96-Hour Static Acute Toxicity Test						
Conducted <u>07/21/09 - 07/25/09</u>		<u>Using: Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4</u>						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	>1125	652	386	371
71 (65) mg/L	0	0	5	5	96-Hour LC ₅₀ * = 371 mg/L			
141 (132) mg/L	0	0	5	10				
	LC ₅₀ 95% Confidence Limits							
282 (272) mg/L	0	0	5	5	24-Hr	48-Hr	72-Hr	96-Hr
564 (569) mg/L	0	40	100	100	LL NR	508	NR	323
					UL NR	838	NR	426
1129 (1125) mg/L	0	85	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 20. Analytical chemistry data for 96-hour toxicity test on *Sphaerium simile* with nitrate.

Nominal (Measured) Test Concentration		N-NO ₂ ^a (mg/L)	N-NO ₃ ^{a,b} (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	<MDL	<MDL	22.5	8.0	8.03	305	60	90
	Day 1			22.8		7.33			
	Day 2			22.9		7.65			
	Day 3			22.8		7.36			
	Day 4	<MDL	0.09	22.9	8.1	7.54	317	60	90
			na						
71 (65) mg/L	Day 0	<MDL	65	22.7	8.0	8.23	883	60	90
	Day 1			22.9		7.19			
	Day 2			22.9		7.54			
	Day 3			22.9		7.44			
	Day 4	<MDL	65	22.9	8.0	7.51	889	60	90
			65						
141 (132) mg/L	Day 0	<MDL	132	22.6	8.0	8.28	1447	60	90
	Day 1			22.9		7.42			
	Day 2			22.9		7.50			
	Day 3			22.9		7.67			
	Day 4	<MDL	133	22.9	8.0	7.74	1456	60	90
			132						
282 (272) mg/L	Day 0	<MDL	272	22.7	8.0	8.22	2530	60	90
	Day 1			22.9		7.21			
	Day 2			22.9		7.60			
	Day 3			23.0		7.45			
	Day 4	<MDL	272	22.9	8.0	7.81	2550	60	90
			272						
564 (569) mg/L	Day 0	<MDL	560	22.7	8.0	8.31	4650	60	90
	Day 1			22.8		7.56			
	Day 2			22.8		7.70			
	Day 3			22.9		4.90			
	Day 4	0.59	579	22.9	7.9	6.14	4770	62	92
			569						
1129 (1125) mg/L	Day 0	<MDL	1100	22.6	8.0	8.31	8710	60	90
	Day 1			22.7		7.45			
	Day 2			22.8		7.24			
	Day 3			22.9		4.52			
	Day 4	0.24	1150	22.8	7.8	6.44	8920	62	90
			1125						

^a Nitrite Analysis Method 354.1

^b Nitrite + Nitrate Analysis Method 353.1

na = not applicable

96-hr Toxicity of Nitrate on *Hyalella azteca*

The 96-hr test to determine the toxicity of nitrate on *H. azteca* was completed by GLEC. *H. azteca* were collected from GLEC's laboratory culture. These organisms are maintained in 10 gallon glass aquaria; plastic artificial turf and screen mesh serve as a substrate for the culture. The tanks are filled with de-chlorinated Lake Michigan water (City of Traverse City, Michigan water passed through an activated carbon filter). Cultures are fed 50 mL of 4 g/L Tetrafin slurry daily. When visible algae are not observed within the glass aquaria, algae (*Selenastrum* sp.) are used as a supplement to the Tetrafin slurry. Additionally, on occasion, dried Aspen (*Populus* sp.) leaves are prepared as a food supplement. The culture is maintained in a 16-hour light: 8-hour dark photoperiod at a temperature between 23 and 26 °C.

Test organisms were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 1.8, 2.9, 4.9, 8.1, 13.6, and 22.6 mg N-NO₃/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ was with the Probit (48 and 72-hour) and Trimmed Spearman-Kärber (96-hour) methods.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 21; test results are provided in Table 22. Analytical chemistry data are provided in Table 23. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 6.

Table 21. Test conditions for 96-hour toxicity test on *Hyalella azteca* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Hyalella azteca</i> , 10 days old, GLEC culture
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	August 6-August 10, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	100 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg N-NO ₃ /L):	22.6, 13.6, 8.1, 4.9, 2.9, and 1.8
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg N-NO ₃ /L):	25.8, 15.2, 9.1, 5.7, 3.2, and 2.1
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USPEA MHRW
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent Cas. No. 7631-99-4, Batch # 66696LJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 22. Test results for 96-hour toxicity test on *Hyalella azteca* with nitrate.

Results of a <i>Hyalella azteca</i> 96-Hour Static Acute Toxicity Test Conducted 08/06/09 - 08/10/09 Using: Nitrate (Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ *Values (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	>25.8	28	23	16.4
1.8 (2.1) mg/L	0 (0)	10 (10)	10 (10)	10 (10)	96-Hour LC₅₀* = 16.4 mg/L			
2.9 (3.2) mg/L	0 (0)	0 (0)	5 (5)	10 (10)				
					LC₅₀ 95% Confidence Limits			
4.9 (5.7) mg/L	0 (5)	0 (0)	0 (0)	15 (15)	24-Hr	48-Hr	72-Hr	96-Hr
8.1 (9.1) mg/L	0 (10)	15 (15)	15 (15)	20 (20)	LL NA	20.7	17.3	13.3
					UL NA	56.2	38.9	20.0
13.6 (15.2) mg/L	10 (15)	10 (10)	20 (20)	25 (25)	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
22.6 (25.8) mg/L	30 (60)	50 (75)	65 (65)	90 (90)				
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Trimmed Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 23. Analytical chemistry data for 96-hour toxicity test on *Hyaella azteca* with nitrate.

Nominal (and Measured) Test Concentrations		Nitrate ^b (mg NO ₃ -N/L)	Nitrite ^a (mg NO ₂ -N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.0011	<0.0011	22.0	8.00	8.4	300	60	80	ND
	Day 1			22.0	7.98	8.5				
	Day 2			22.7	8.26	8.2				
	Day 3			22.3	8.07	8.2				
	Day 4	0.0443	0.0201	21.9	7.89	8.0	290	60	80	
		0.0560	0.0208							
		0.050								
1.8 (2.1) mg/L	Day 0	2.13	<0.0011	22.0	8.06	8.4	352			
	Day 1	2.16		22.0	7.99	8.6				
	Day 2			22.7	8.25	8.7				
	Day 3			22.5	8.02	8.2				
	Day 4	2.15	0.0062	22.1	7.84	8.0	327			
		2.1								
2.9 (3.2) mg/L	Day 0	2.93	<0.0011	22.0	8.08	8.4	362			
	Day 1			22.1	7.95	8.6				
	Day 2			22.7	8.10	8.7				
	Day 3			22.8	8.04	8.3				
	Day 4	3.40	0.0067	22.2	7.84	8.0	330			
		3.2								
4.9 (5.7) mg/L	Day 0	5.68	<0.0036	22.0	8.10	8.4	377			
	Day 1			22.1	7.97	8.6				
	Day 2			22.7	8.09	8.8				
	Day 3			22.9	8.09	8.5				
	Day 4	5.77	0.0051	22.2	7.80	8.0	356			
		5.7								
8.1 (9.1) mg/L	Day 0	9.76	<0.0011	22.0	8.07	8.4	402			
	Day 1			22.1	8.00	8.6				
	Day 2			22.7	8.08	8.9				
	Day 3			22.9	8.11	8.7				
	Day 4	8.52	0.0062	22.2	7.80	8.0	390			
		9.1								
13.6 (15.2) mg/L	Day 0	15.52	<0.0011	22.0	8.07	8.4	443			
	Day 1			22.1	8.02	8.5				
	Day 2			22.7	8.09	9.0				
	Day 3			22.9	8.07	8.7				
	Day 4	14.89	0.4104	22.2	7.80	8.0	400			
		15.2								
22.6 (25.8) mg/L	Day 0	27.45	<0.0011	22.0	8.04	8.4	513	60	80	ND
	Day 1			22.1	8.01	8.5				
	Day 2			22.7	8.06	9.0				
	Day 3			22.9	8.06	8.9				
	Day 4	24.22	0.0133	22.1	7.84	8.0	500	60	84	
		25.8								

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM 4500 NO₃

ND Not Detect; below detection limit

96-hr Toxicity of Nitrate on *Pimephales promelas*

The 96-hr test to determine the toxicity of nitrate on *P. promelas* was completed by GLEC. Test organisms, collected from the GLEC laboratory culture, were acclimated to the dilution water (de-chlorinated Lake Michigan water), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); two replicates were used per treatment with ten organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 87.9, 146, 244, 407 and 678 mg N-NO₃/L.

Testing was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 24; test results are provided in Table 25. Analytical chemistry data are provided in Table 26. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 7.

Table 10. Test conditions for 96-hour toxicity test on *Pimephales promelas* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (weight 0.11 g and length 16 mm), GLEC culture
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	August 6-August 10, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	4000 mL beaker
9. Volume of Test Solutions:	3500 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg N-NO ₃ /L):	678, 407, 244, 146, and 87.9
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg N-NO ₃ /L):	720, 406, 262, 160, and 98
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent Cas. No. 7631-99-4, Batch # 66696LJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 25. Test results for 96-hour toxicity test on *Pimephales promelas* with nitrate.

Results of a <u>Pimephales promelas</u> <u>96-Hour Static Acute Toxicity Test</u>								
Conducted <u>08/06/09 - 08/10/09</u> <u>Using: Nitrate (Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4)</u>								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ *Values (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	>720	580	536	415
88 (98) mg/L	0 (0)	0 (0)	0 (0)	0 (0)	96-Hour LC ₅₀ * = 415 mg/L			
146 (160) mg/L	0 (0)	0 (0)	0 (0)	5 (5)	LC ₅₀ * 95% Confidence Limits			
244 (262) mg/L	0 (0)	20 (20)	20 (20)	25 (25)	24-Hr	48-Hr	72-Hr	96-Hr
407 (406) mg/L	0 (0)	20 (20)	25 (25)	50 (50)	LL NA	465	437	343
678 (720) mg/L	35 (35)	65 (65)	70 (70)	80 (80)	UL NA	841	730	526
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 26. Analytical chemistry data for 96-hour toxicity test on *Pimephales promelas* with nitrate.

Nominal (and Measured) Test Concentration		Nitrate ^b (mg NO3-N/L)	Nitrite ^a (mg NO2-N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)		Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	0.2528	<0.0011	25	8.04	8.4	8.4	310	96	136	ND
	Day 1			24.6	7.71	5.9	6.0				
	Day 2			24.3	7.81	6.6	7.4				
	Day 3			24.4	8.28	6.7	6.9				
	Day 4	0.2460	0.0028	24.4	8.00	7.0	7.0	302	100	140	
		0.2345		24.4	8.04						
		0.2444									
88 (98) mg/L	Day 0	97.5830	<0.0011	25.0	8.10	8.4	8.4	1030			
	Day 1			24.6	7.68	5.9	5.7				
	Day 2			24.2	7.77	7.7	6.3				
	Day 3			24.3	8.11	6.7	6.9				
	Day 4	97.5830	0.0113	24.4	8.12	6.8	7.0	979			
		97.5830		24.3	8.14						
146 (160) mg/L	Day 0	162.1866	<0.0011	25.0	8.09	8.4	8.4	1483			
	Day 1			24.6	7.68	5.8	5.8				
	Day 2			24.2	7.75	7.9	6.6				
	Day 3			24.4	8.11	6.9	6.8				
	Day 4	156.3135	0.0218	24.3	8.10	6.6	6.7	1429			
		160.6054	0.0205	24.4	8.14						
		159.7018									
244 (262) mg/L	Day 0	266.7721	0.0026	25.0	8.07	8.4	8.4	2241			
	Day 1			24.6	7.68	5.8	5.7				
	Day 2			24.3	7.65	5.9	5.4				
	Day 3			24.4	8.10	6.5	6.4				
	Day 4	256.3813	0.0883	24.4	8.05	6.7	6.9	2170			
		261.5767		24.4	8.03						
407 (406) mg/L	Day 0	410.2101	0.0026	25.0	8.07	8.4	8.4	3361			
	Day 1			24.6	7.57	5.9	5.8				
	Day 2			24.4	7.47	6.7	6.0				
	Day 3			24.3	8.00	6.2	6.0				
	Day 4	402.5299	0.1096	24.4	7.94	6.4	6.6	3250			
		406.3700		24.4	8.02						
678 (720) mg/L	Day 0	754.0095	<0.0011	25.0	8.05	8.4	8.4	5550	100	136	ND
	Day 1		<0.0011	24.6	7.47	5.9	5.6				
	Day 2			24.4	7.42	5.9	5.9				
	Day 3			24.3	7.88	6.6	6.7				
	Day 4	685.5658	0.1428	24.4	8.03	6.5	6.6	5240	104	140	
		719.6747		24.4	7.94						

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM 4500 NO₃

ND Not Detect; below detection limit

32-day Toxicity of Nitrate on *Pimephales promelas*

The 32-day test to determine the toxicity of nitrate on *P. promelas* was completed by GLEC. The fish were continuously exposed for 32 days to five concentrations of nitrate (nominal concentrations of 28.2, 56.5, 113, 226 and 452 mg N-NO₃/L) and to a dilution water control using a continuous flow-through system (Benoit et al. 1982). The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were four replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with < 24 hour old fertilized embryos. The embryos were randomly assigned to incubation cups until each incubation cup contained 30 embryos. The incubation cups were randomly assigned to the 2.5 L glass test chambers (1 cup per chamber) and suspended in the test solutions from a rocker arm assembly. The rocker arm assembly moves the incubation cups in a reciprocal motion within each test chamber. Embryos were inspected on a daily basis and the number of live, hatched and dead embryos was recorded. On Day 5 of the test (three days after first hatch), the surviving fish were randomly thinned to achieve 20 fish in each test chamber. The remainder of the surviving fish was discarded. The number of surviving fish was recorded at test termination (32 days). In addition, the wet weights were recorded for each fish at test termination. Because of the size range of fish in each test chamber, all of the fish from each test chamber were weighed together to determine average dry weight.

There were several instances throughout the test when instantaneous water temperature measurements made within individual replicates exceeded those allowed in the toxicity testing method. However, incidences of temperature exceedance only occurred intermittently and the average water temperatures (across the duration of the test) in each replicate were always ± 0.4 °C of the target test temperature (25 °C) in all treatments. In addition, the water temperature as recorded by the continuous temperature logger was never outside the range outlined in the method. Therefore, these water temperature exceedances likely had no effect on the results of this toxicity test.

In addition, there was an instance of low DO (4.3 mg/L) in one of the replicates at test termination. Because this incident occurred on the last day of the test and similarly low DO concentrations were never observed on any other day of the test, this low DO was an anomaly and likely had no effect on the results of this toxicity test.

Once the test was complete, the LC₅₀, NOEC, and LOEC values were determined using the average measured concentrations with the Spearman Karber and ANOVA methods. LC₂₅, LC₂₀, LC₁₀, EC₅₀, EC₂₅, EC₂₀ and EC₁₀ values were estimated using EPA's TRAP.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 27; test results are provided in Table 28. Survival and growth data are provided

in Table 29 and analytical chemistry data are provided in Table 30. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 8.

Table 27. Test conditions for 32-day toxicity test on *Pimephales promelas* with nitrate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (<24 hour fertilized embryos), GLEC culture
2. Test Type and Duration:	Continuous flow-through; 32 days
3. Test Dates:	July 24-August 25, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	Live Brine Shrimp (<i>Artemia nauplii</i>) Twice daily
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume of Test Solutions:	2,000 mL
10. No. of Test Organisms per Test Vessel:	30 eggs, thinned to 20 larvae after hatch
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	120 eggs, thinned to 80 larvae after hatch
13. Target or Nominal Test Concentrations (mg N-NO ₃ /L):	452, 226, 113, 56.5, and 28.2
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg N-NO ₃ /L):	441, 226, 109, 48.6, and 21.7
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Sodium Nitrate: Sigma Aldrich, ≥ 99.0%, ACS Reagent Cas. No. 7631-99-4, Batch # 66696LJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Survival (LC ₅₀ , LC ₂₅ , LC ₂₀ and LC ₁₀ , NOEC and LOEC) and Growth (EC ₅₀ , EC ₂₅ , EC ₂₀ and EC ₁₀ , NOEC and LOEC)

Table 28. Test results for 32-day toxicity test on *Pimephales promelas* with nitrate.

Results of a <i>Pimephales promelas</i> 32-Day Continuous Flow Chronic Toxicity Test Conducted 07/24/09 - 08/25/09 Using: Nitrate (Sodium Nitrate: Sigma Aldrich Cas. No. 7631-99-4)						
Test Solution Concentrations Measured	Primary Control/ Dilution Water	22 mg/L	49 mg/L	109 mg/L	226 mg/L	441 mg/L
Embryo Percent Hatch (%)	100	100	100	99	98	99
32-Day Mean Survival (%)	88.8	86.3	92.5	16.3 ^a	10.0 ^a	0 ^a
32-Day Average Biomass ¹ (mg)	5.44	5.72	5.54	1.91 ^a	0.84 ^a	0 ^a
Embryo Percent Hatch NOEC:	441 mg/L		32-Day LC ₂₀ :	64.6 mg/L (44.7 mg/L – 93.5 mg/L)		
32-Day LC ₅₀ *:	76.8 mg/L (70 mg/L-84 mg/L)		32-Day LC ₁₀ :	55.5 mg/L (34.7 mg/L – 88.7 mg/L)		
32-Day Survival NOEC:	49 mg/L		32-Day EC ₅₀ :	91.3 mg/L (72.9 mg/L – 114.3 mg/L)		
32-Day Survival LOEC:	109 mg/L		32-Day EC ₂₅ :	65.3 mg/L (44.4 mg/L – 96.1 mg/L)		
32-Day Growth NOEC:	49 mg/L		32-Day EC ₂₀ :	59.8 mg/L (38.7 mg/L – 92.4 mg/L)		
32-Day Growth LOEC:	109 mg/L		32-Day EC ₁₀ :	46.7 mg/L (26.2 mg/L – 83.4 mg/L)		
32-Day LC ₂₅ :	68.2 mg/L (48.8 mg/L – 95.4 mg/L)					

^a: Significantly different from dilution water control ($p \leq 0.05$)

*: All LC, EC, NOEC and LOEC values are determined based on the average measured nitrate concentration.

NOEC: No-Observed-Effect-Concentration

LOEC: Lowest-Observed-Effect-Concentration

¹Biomass: Biomass is the average dry weight of the four replicates calculated by the total dry weight of surviving organisms divided by the initial number of organisms (20).

Table 29. Survival and growth data for 32-day toxicity test on *Pimephales promelas* with nitrate.

Nominal (and Measured) Test Concentration		Number of Eggs at Test Initiation	Number of Hatched Larvae	Number of Dead Eggs	Percent Hatched Larvae	Number of Larvae at Test Termination	Percent Survival at Test Termination*	Biomass ¹ (mg)
Dilution water/Control	Replicate # 1	30	30	0	100.0	18	90.0	5.34
	Replicate # 2	30	30	0	100.0	18	90.0	5.94
	Replicate # 3	30	30	0	100.0	20	100.0	5.65
	Replicate # 4	30	30	0	100.0	15	75.0	4.79
	Average				100.0		88.8	5.43
28 (22) mg/L	Replicate # 1	30	30	0	100.0	18	90.0	6.62
	Replicate # 2	30	30	0	100.0	15	75.0	5.67
	Replicate # 3	30	30	0	100.0	18	90.0	5.34
	Replicate # 4	30	30	0	100.0	18	90.0	5.26
	Average				100.0		86.3	5.72
56 (49) mg/L	Replicate # 1	30	30	0	100.0	19	95.0	5.45
	Replicate # 2	30	30	0	100.0	20	100.0	5.98
	Replicate # 3	30	30	0	100.0	20	100.0	5.25
	Replicate # 4	30	30	0	100.0	15	75.0	5.46
	Average				100.0		92.5	5.54
113 (109) mg/L	Replicate # 1	30	30	0	100.0	3	15.0	1.78
	Replicate # 2	30	29	1	96.7	4	20.0	1.84
	Replicate # 3	30	30	0	100.0	4	20.0	2.79
	Replicate # 4	30	30	0	100.0	2	10.0	1.25
	Average				99.2		16.3	1.91
226 (226) mg/L	Replicate # 1	30	29	1	96.7	1	5.0	0.20
	Replicate # 2	30	30	0	100.0	4	20.0	2.02
	Replicate # 3	30	29	1	96.7	2	10.0	1.04
	Replicate # 4	30	30	0	100.0	1	5.0	0.10
	Average				98.3		10.0	0.84
452 (441) mg/L	Replicate # 1	30	29	1	96.7	0	0.0	0.00
	Replicate # 2	30	30	0	100.0	0	0.0	0.00
	Replicate # 3	30	30	0	100.0	0	0.0	0.00
	Replicate # 4	30	30	0	100.0	0	0.0	0.00
	Average				99.2		0.0	0.00

* On Day 5 of the test, (three days after first hatch) the surviving fish were randomly thinned to 20 fish in each test chamber. Percent survival at test termination is the number of surviving fish at test termination divided by 20.

¹ Biomass: Biomass is the total dry weight of surviving organisms divided by the initial number of organisms (20)

Table 30. Analytical chemistry data for 32-day toxicity test on *Pimephales promelas* with nitrate.

Nominal (and Measured) Test Concentrations		NO ₃ (mg NO ₃ -N/L)	NO ₂ (mg NO ₂ -N/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmhos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate # 1	0.17 (0.005-0.258)	0.006	25.3 (24.0-26.3)	7.97	7.4 (7.2-7.8)	298	180	93	ND
	Replicate # 2	0.26 (0.221-0.307)	0.003	25.0 (24.2-26.0)	8.13	7.6 (7.2-8.2)	296			ND
	Replicate # 3	0.26 (0.226-0.289)	0.003	25.0 (24.1-25.6)	8.17	7.7 (7.3-8.2)	295			ND
	Replicate # 4	0.24 (0.210-0.271)	0.003	25.0 (24.2-26.1)	8.24	7.7 (7.2-8.1)	296			ND
	Average	0.23								
28 mg/L (22 mg/L)	Replicate # 1	20 (12-40)	0.006	24.9 (24.0-25.8)	8.21	7.6 (6.7-8.4)	442	154	104	ND
	Replicate # 2	22 (13-41)	0.003	24.9 (24.1-26.0)	8.22	7.7 (7.1-8.2)	483			ND
	Replicate # 3	21 (14-41)	0.006	25.0 (24.2-25.7)	8.27	7.8 (7.3-9.2)	426			ND
	Replicate # 4	24 (13-41)	0.006	25.1 (24.2-25.7)	8.32	7.9 (7.2-9.2)	418			ND
	Average	22								
56 mg/L (49mg/L)	Replicate # 1	48 (38-65)	0.009	25.0 (24.2-26.0)	8.23	7.6 (6.6-9.1)	626	140	107	ND
	Replicate # 2	48 (37-67)	0.006	24.9 (24.1-25.8)	8.22	7.8 (7.4-8.1)	689			ND
	Replicate # 3	51 (38-69)	0.006	25.1 (24.3-26.1)	8.16	7.6 (7.1-8.0)	654			ND
	Replicate # 4	47 (39-70)	0.006	25.1 (24.2-26.0)	8.23	7.8 (7.0-9.0)	667			ND
	Average	49								
113 mg/L (109 mg/L)	Replicate # 1	107 (90-146)	0.009	24.8 (24.0-26.0)	8.27	7.6 (6.8-8.2)	1045	164	101	ND
	Replicate # 2	112 (95-141)	0.003	24.6 (23.7-26.0)	8.24	7.7 (7.0-8.3)	1083			ND
	Replicate # 3	107 (88-137)	0.003	24.9 (24.2-25.6)	8.26	7.9 (7.4-9.4)	984			ND
	Replicate # 4	109 (92-139)	0.006	24.8 (23.7-25.5)	8.26	7.8 (7.1-9.3)	958			ND
	Average	109								
226 mg/L (226 mg/L)	Replicate # 1	220 (157-285)	0.006	24.9 (24.2-26.0)	8.24	7.6 (6.9-8.0)	2449	132	104	ND
	Replicate # 2	232 (168-300)	0.225	24.7 (23.6-25.9)	8.10	7.2 (4.3-8.0)	2206			ND
	Replicate # 3	225 (172-297)	0.006	25.0 (24.2-25.8)	8.25	7.7 (6.6-9.4)	2137			ND
	Replicate # 4	227 (158-297)	0.006	25.0 (24.0-25.9)	8.26	7.8 (6.8-9.4)	2240			ND
	Average	226								
452 mg/L (441 mg/L)	Replicate # 1	453 (389-485)	0.006	25.1 (24.2-26.0)	8.22	7.5 (6.4-8.1)	3820	146	101	ND
	Replicate # 2	421 (341-471)	0.006	24.8 (23.9-26.0)	8.23	7.7 (6.9-8.3)	3763			ND
	Replicate # 3	452 (411-493)	0.033	24.8 (24.0-25.7)	8.15	7.5 (6.8-9.4)	4001			ND
	Replicate # 4	437 (411-463)	0.009	25.0 (24.1-25.8)	8.19	7.8 (6.2-9.4)	3610			ND
	Average	441								

DO: Dissolved Oxygen
SC: Specific Conductance
ND: Not detected

Nitrite

Table 31 provides a summary of estimated LC₅₀ values for the nine toxicity tests performed using nitrite. LC₅₀ values ranged between 1.0 and 177 mg NO₃-N/L.

Table 31. LC₅₀ estimates for toxicity tests performed using nitrite.

Test Species and Duration	LC ₅₀ (mg NO ₂ -N/L)
<i>Ceriodaphnia dubia</i> - 48 hr	4.6
<i>Lampsilis siliquoidea</i> - 96 hr	177
<i>Chironomus dilutus</i> - 48 hr	15.6
<i>Amphinemura delosa</i> - 96 hr	1.0
<i>Sphaerium simile</i> - 96 hr	55.7
<i>Hyalella azteca</i> - 96 hr	1.5
<i>Pimephales promelas</i> - 96 hr	27.0
<i>Pimephales promelas</i> - 32 day	11.3
<i>Ceriodaphnia dubia</i> - 7 day	4.7

For each of the acute toxicity tests completed using nitrite, two tables were generated: the first summarizes the test results for each toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity tests. The results of chronic tests performed with nitrite were summarized in three tables: the first summarizes nominal and analytical test concentrations, LC₅₀ estimates with confidence intervals, NOEC and LOEC estimates, mean survival and mean biomass; the second table summarizes replicate-specific survival and growth data and the third table summarizes analytical chemistry data collected throughout the toxicity tests. Also discussed, if applicable, are deviations from the guidance provided in the ASTM method used to complete the toxicity testing.

Nitrate and nitrite concentration data generated by the GLEC analytical chemistry laboratory were reported as nitrate (mg NO₃/L) and nitrite (mg NO₂/L). For consistency (i.e. so that the data correspond to the nitrate and nitrogen data generated by the analytical chemistry laboratory used by the INHS), the GLEC data were converted to nitrate-nitrogen (N-NO₃/L) and nitrite-nitrogen (N-NO₂/L) for LC₅₀ determinations and reporting purposes. The data as reported by the GLEC analytical laboratory are available in the appendices.

96-hr Toxicity of Nitrite on *Lampsilis siliquoidea*

The 96-hr test to determine the toxicity of nitrite on *L. siliquoidea* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Missouri State University laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 304, 152, 76, 38, 19, and 9.5 mg N-NO₂/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 32; test results are provided in Table 33. Analytical chemistry data are provided in Table 34. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 9.

Table 32. Test conditions for 96-hour toxicity test on *Lampsilis siliquoidea* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Lampsilis siliquoidea</i> , juveniles <5 days old, Missouri State University
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 08 - 12, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume and Depth of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg N-NO ₂ /L):	304, 152, 76, 38, 19, and 9.5
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg N-NO ₂ /L):	312.8, 158.1, 82.0, 39.1, 19.6, and 9.9
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrite: Fisher, certified ACS, assay 99.7%, Lot # 080939
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 33. Test results for 96-hour toxicity test on *Lampsilis siliquoidea* with nitrite.

Results of a <i>Lampsilis siliquoidea</i> 96-Hour Static Acute Toxicity Test Conducted 09/08/09 - 09/12/09 Using: Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L) (EC ₅₀ *)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	>312.8	>312.8	240.6	177.0
9.5 (9.9) mg/L	0	0	0	10	96-Hour LC₅₀* = 177.0 mg/L			
19.0 (19.6) mg/L	0	0	5	10				
					LC ₅₀ 95% Confidence Limits			
38.0 (39.1) mg/L	0	0	0	0	24-Hr	48-Hr	72-Hr	96-Hr
76.0 (82.0) mg/L	0	0	0	10	LL NR	NR	209.9	145.0
					UL NR	NR	275.7	215.0
152.0 (158.1) mg/L	0	0	10	30	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
304.0 (312.8) mg/L	0	0	75	100				
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 34. Analytical chemistry data for 96-hour toxicity test on *Lanpsilis siliquoides* with nitrite.

Nominal (Measured) Test Concentration		N-NO ₂ ^a (mg/L)	N-NO ₃ ^{a,b} (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	<MDL	NM	20.2	8.0	7.79	307	62	88
	Day 1			20.2					
	Day 2			20.3					
	Day 3			19.6					
	Day 4	<MDL	NM	19.2	7.9	7.70	310	64	90
		na							
9.5 (9.9) mg/L	Day 0	9.7	NM	20.2	8.0	7.77	388	66	88
	Day 1			20.3					
	Day 2			20.3					
	Day 3			19.6					
	Day 4	10.1	NM	19.2	7.9	7.71	390	66	90
		9.6							
19.0 (19.6) mg/L	Day 0	18.9	NM	20.2	8.0	7.78	469	66	88
	Day 1			20.4					
	Day 2			20.3					
	Day 3			19.6					
	Day 4	20.4	NM	19.2	7.9	7.68	476	66	90
		19.6							
38.0 (39.1) mg/L	Day 0	38.4	NM	20.2	8.0	7.77	633	68	88
	Day 1			20.4					
	Day 2			20.3					
	Day 3			19.6					
	Day 4	39.9	NM	19.3	7.9	7.80	640	68	90
		39.1							
76.0 (82.0) mg/L	Day 0	80.8	NM	20.2	8.0	7.77	947	68	88
	Day 1			20.3					
	Day 2			20.4					
	Day 3			19.7					
	Day 4	83.3	NM	19.3	7.9	7.65	955	66	90
		82.0							
152.0 (158.1) mg/L	Day 0	155.0	NM	20.2	8.0	7.72	1566	92	88
	Day 1			20.4					
	Day 2			20.4					
	Day 3			19.7					
	Day 4	161.3	NM	19.4	7.9	7.79	1580	94	90
		158.1							
304.0 (312.8) mg/L	Day 0	309.7	NM	20.2	8.0	7.73	2750	120	90
	Day 1			20.4					
	Day 2			20.5					
	Day 3			19.7					
	Day 4	316.0	NM	19.4	7.9	7.71	2760	120	90
		312.8							

^a Nitrite Analysis Method 354.1 NM = Not measured

^b Nitrite + Nitrate Analysis Method 353.1 na = not applicable

48-hr Toxicity of Nitrite on *Chironomus dilutus*

The 48-hr test to determine the toxicity of nitrite on *C. dilutus* was completed by GLEC. Serial dilutions. Test organisms, 10-days old from the GLEC culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 2.4, 3.9, 6.6, 11, 18 and 30 mg NO₂-N/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 35; test results are provided in Table 36. Analytical chemistry data are provided in Table 37. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 10.

Table 35. Test conditions for 48-hour toxicity test on *Chironomus dilutus* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Chironomus dilutus</i> , 10 days old, GLEC culture
2. Test Type and Duration:	Static, 48 hours
3. Test Dates:	July 23-July 25, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	100 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg NO ₂ -N/L):	30, 18, 11, 6.6, 3.9, and 2.4
14. Measured Test Concentrations (average of samples collected at test initiation and termination- mg NO ₂ -N/L):	32.4, 19, 11, 6.8, 4.0, and 2.6
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrite: Sigma Aldrich, 97 +%, ACS Reagent Cas. No. 7632-00-0, Batch # 08316DJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 36. Test results for 48-hour toxicity test on *Chironomus dilutus* with nitrite.

Results of a <u><i>Chironomus dilutus (tentans)</i></u> <u>48-Hour Static Acute Toxicity Test</u>								
Conducted <u>07/23/09 - 07/25/09</u> Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)			31.2	15.6	NA	NA
2.4 (2.6) mg/L	0 (0)	0 (0)			48-Hour LC ₅₀ * = 15.6 mg/L			
3.9 (4.0) mg/L	0 (0)	0 (0)						
					LC ₅₀ 95% Confidence Limits (mg/L)			
6.6 (6.8) mg/L	0 (0)	10 (10)			24-Hr	48-Hr	72-Hr	96-Hr
11 (11) mg/L	0 (0)	20 (20)			LL 25.4	13.2	NA	NA
					UL 47.5	18.9	NA	NA
18 (19) mg/L	20 (20)	50 (50)			LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
30 (32) mg/L	50 (50)	100 (100)						
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 37. Analytical chemistry data for 48-hour toxicity test on *Chironomus dilutus* with nitrite.

Nominal (and Measured) Test Concentrations		Nitrite ^a (mg NO ₂ -N/L)	Nitrate ^b (mg NO ₃ -N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.0011	0.000	22.3	8.04	7.4	292	60	84	ND
			0.000							
	Day 1			21.4	7.78	7.5				
	Day 2	0.008	<0.0012	22.4	7.80	7.7	290	90	124	ND
		0.008								
2.4 (2.6) mg/L	Day 0	2.7	0.400	22.7	8.01	7.5	291			
		2.6								
	Day 1			21.4	7.80	7.5				
	Day 2	2.5	0.115	22.4	7.81	7.7	290			
		2.6								
3.9 (4.0) mg/L	Day 0	3.9	0.097	22.4	8.02	7.6	330			
	Day 1			21.4	7.84	7.5				
	Day 2	4.1	3.885	22.4	7.85	7.5	334			
		4.0								
6.6 (6.8) mg/L	Day 0	6.7	0.700	22.5	8.05	7.6	381			
	Day 1			21.4	7.89	7.5				
	Day 2	6.5	0.339	22.5	7.88	7.5	394			
		7.1	<0.1129							
		6.8								
11 (11) mg/L	Day 0	10.7	0.971	22.5	8.05	7.6	436			
	Day 1			21.4	7.89	7.6				
	Day 2	10.7	0.542	22.3	7.91	7.2	430			
		10.7								
18 (19) mg/L	Day 0	18.3	3.275	22.4	8.05	7.5	478			
	Day 1			21.4	7.91	7.7				
	Day 2	18.9	<0.2485	22.3	7.91	7.2	484			
		18.6								
30 (32) mg/L	Day 0	32.9	< 1.13	22.5	8.03	7.7	525	60	80	ND
	Day 1			21.4	7.95	7.7				
	Day 2	32.0	0.949	22.3	7.99	7.3	530	64	116	ND
		32.4								

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM 4500 NO₃

ND Not Detected; below detection limit

96-hr Toxicity of Nitrite on *Amphinemura delosa*

The 96-hr test to determine the toxicity of nitrite on *A. delosa* was completed by INHS. Test organisms, field-collected nymphs, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 12, 6.1, 3.0, 1.5, and 0.8 mg N-NO₂/L.

Testing was conducted at 12 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 38; test results are provided in Table 39. Analytical chemistry data are provided in Table 40. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 11.

Table 38. Test conditions for 96-hour toxicity test on *Amphinemura delosa* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Amphinemura delosa</i> , nymphs, Field collected
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	May 14 - 18, 2009
4. Test Temperature (°C):	12 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	250 mL beaker
9. Volume of Test Solutions:	200 mL
10. No. of Test Organisms per Test Vessel:	4
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	16
13. Test Concentrations (mg N-NO ₂ /L):	12, 6.1, 3.0, 1.5, and 0.8
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg N-NO ₂ /L):	13, 6.1, 3.1, 1.5, and 0.7
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrite: Fisher, certified ACS, assay 99.7%, Lot # 080939
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 39. Test results for 96-hour toxicity test on *Amphinemura delosa* with nitrite.

Results of a <u><i>Amphinemura delosa</i></u>		<u>96-Hour Static Acute Toxicity Test</u>						
Conducted <u>05/14/09 - 05/18/09</u>		<u>Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)</u>						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L) (EC ₅₀ *)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	6.25	6.25	6.25	3.6	1.7	1.0	1.0
0.8 (0.7) mg/L	12.5	12.5	18.75	25	96-Hour LC ₅₀ * = 1.0 mg/L			
1.5 (1.5) mg/L	12.5	31.25	81.25	81.25				
					LC ₅₀ 95% Confidence Limits			
3.0 (3.1) mg/L	50	100	100	100	24-Hr	48-Hr	72-Hr	96-Hr
6.1 (6.1) mg/L	68.75	100	100	100	LL 2.2	1.3	0.9	0.8
					UL 5.8	2.2	1.2	1.2
12.2 (12.7) mg/L	75	100	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 40. Analytical chemistry data for 96-hour toxicity test on *Amphinemura delosa* with nitrite.

Nominal (Measured) Test Concentration		N-NO2 ^a	N-NO3 ^{a,b}	Temperature	pH	D.O.	Conductivity	Alkalinity	Hardness
		(mg/L)	(mg/L)	(°C)	(s.u.)	(mg/L)	(µmhos)	(mg/L)	(mg/L)
Dilution water/Control	Day 0	<0.08	<0.08	12.5	7.9	9.10	301	60	88
	Day 1			12.1	7.9		300		
	Day 2			12.3	7.9		300		
	Day 3			12.5	7.9		305		
	Day 4	<0.07	<0.07	12.8	7.9	9.97	299	60	90
		na							
0.8 (0.7) mg/L	Day 0	0.8	1.0	12.5	7.8	9.17	306	60	90
	Day 1			12.1	7.9		303		
	Day 2			12.3	7.9		302		
	Day 3			12.5	7.9		303		
	Day 4	0.7	1.0	12.7	7.9	9.98	307	60	90
		0.7							
1.5 (1.5) mg/L	Day 0	1.5	2.1	12.5	7.8	9.13	314	60	90
	Day 1			12.1	7.9		310		
	Day 2			12.3	7.9		310		
	Day 3			12.5	7.9		310		
	Day 4	1.5	2.0	12.8	7.9	9.93	313	60	90
		1.5							
3.0 (3.1) mg/L	Day 0	3.1	4.1	12.5	7.8	9.14	327	60	90
	Day 1			12.3	7.9		324		
	Day 2			12.3	7.9		325		
	Day 3			12.5	7.9		325		
	Day 4	3.0	3.8	12.7	7.9	9.97	324	60	90
		3.1							
6.1 (6.1) mg/L	Day 0	6.1	8.9	12.5	7.8	9.06	354	60	90
	Day 1			12.3	7.9		351		
	Day 2			12.3	7.9		355		
	Day 3			12.5	7.9		350		
	Day 4	6.1	3.6	12.7	7.9	9.99	351	60	88
		6.1							
12.2 (12.7) mg/L	Day 0	12.8	18.6	12.5	7.8	9.08	406	60	90
	Day 1			12.2	7.9		404		
	Day 2			12.3	7.9		405		
	Day 3			12.5	7.9		405		
	Day 4	12.7	14.5	12.2	7.9	9.99	405	62	90
		12.7							

^a Nitrite Analysis Method 354.1

^b Nitrite + Nitrate Analysis Method 353.1

na = not applicable

96-hr Toxicity of Nitrite on *Sphaerium simile*

The 96-hr test to determine the toxicity of nitrite on *S. simile* was completed by INHS. Test organisms, juveniles released from field-collected adults, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 304, 152, 76, 38, 19, and 9.5 mg N-NO₂/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 41; test results are provided in Table 42. Analytical chemistry data are provided in Table 42. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 12.

Table 41. Test conditions for 96-hour toxicity test on *Sphaerium simile* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Sphaerium simile</i> , juveniles (released from field-collected adults)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	August 17-21, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	120 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg N-NO ₂ /L):	304, 152, 76, 38, 19, and 9.5
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg N-NO ₂ /L):	296, 158, 82, 39, 19, and 9.4
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrite: Fisher, certified ACS, assay 99.7%, Lot # 080939
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 42. Test results for 96-hour toxicity test on *Sphaerium simile* with nitrite.

Results of a <i>Sphaerium simile</i> 96-Hour Static Acute Toxicity Test								
Conducted 08/17/09 - 08/21/09 Using: Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	>296.1	240.0	178.7	55.7
9.5 (9.4) mg/L	0	0	0	0	96-Hour LC ₅₀ * = 55.7 mg/L			
19.0 (19.2) mg/L	0	0	0	15				
					LC ₅₀ 95% Confidence Limits			
38.0 (38.8) mg/L	0	0	0	40	24-Hr	48-Hr	72-Hr	96-Hr
76.1 (82.2) mg/L	0	0	0	50	LL NR	171.1	65.1	43.0
					UL NR	336.7	490.7	72.1
152.2 (157.7) mg/L	0	30	50	95	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
304.4 (296.1) mg/L	0	60	60	100				
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 43. Analytical chemistry data for 96-hour toxicity test on *Sphaerium simile* with nitrite.

Nominal (Measured) Test Concentration		N-NO2 ^a	N-NO3 ^{a,b}	Temperature	pH	D.O.	Conductivity	Alkalinity	Hardness
		(mg/L)	(mg/L)	(°C)	(s.u.)	(mg/L)	(µmhos)	(mg/L)	(mg/L)
Dilution water/Control	Day 0	<0.02	NM	22.3	8.0	7.74	302	62	88
	Day 1			22.9	7.7	7.14	304		
	Day 2			22.7	7.7	7.17	313		
	Day 3			22.7	7.7	7.31	316		
	Day 4	<0.02	NM	22.7	7.7	7.51	317	62	90
		na							
9.5 (9.4) mg/L	Day 0	9.6	NM	22.4	8.1	7.81	383	62	90
	Day 1			22.8	7.7	6.49	384		
	Day 2			22.7	7.3	6.30	389		
	Day 3			22.6	7.6	6.67	393		
	Day 4	9.2	NM	22.7	7.6	6.78	395	64	90
		9.4							
19.0 (19.2) mg/L	Day 0	19.3	NM	22.5	8.1	7.79	466	62	90
		19.8							
	Day 1			22.8	7.7	6.59	465		
	Day 2			22.6	7.3	7.09	470		
	Day 3			22.7	7.6	7.10	474		
	Day 4	19.1	NM	22.7	7.6	6.89	481	64	90
		19.7							
		19.2							
38.0 (38.8) mg/L	Day 0	38.9	NM	22.4	8.2	7.80	626	64	88
	Day 1			22.8	7.7	6.25	623		
	Day 2			22.7	7.4	6.86	629		
	Day 3			22.7	7.6	6.78	633		
	Day 4	38.7	NM	22.7	7.6	6.75	639	66	90
		38.8							
76.1 (82.2) mg/L	Day 0	79.2	NM	22.3	8.2	7.82	941	72	88
	Day 1			22.8	7.6	5.82	941		
	Day 2			22.6	7.5	6.81	948		
	Day 3			22.7	7.6	6.26	958		
	Day 4	85.3	NM	22.7	7.6	6.04	971	76	90
		82.2							
152.2 (157.7) mg/L	Day 0	153.5	NM	22.4	8.2	7.82	1562	96	88
	Day 1			22.8	7.7	7.03	1558		
	Day 2			22.7	7.5	6.02	1574		
	Day 3			22.7	7.6	6.09	1577		
	Day 4	162.1	NM	22.8	7.5	6.09	1587	110	90
		157.7							
304.4 (296.1) mg/L	Day 0	293.1	NM	22.5	8.2	7.82	2750	120	88
	Day 1			22.8	7.7	7.03	2740		
	Day 2			22.7	7.5	5.46	2750		
	Day 3			22.6	7.6	5.76	2750		
	Day 4	299.2	NM	22.8	7.7	5.70	2740	126	90
		296.1							

^a Nitrite Analysis Method 354.1 NM = not measured

^b Nitrite + Nitrate Analysis Method 353.1 na = not applicable

96-hr Toxicity of Nitrite on *Hyaella azteca*

The 96-hr test to determine the toxicity of nitrite on *H. azteca* was completed by GLEC. *H. azteca* were collected from GLEC's laboratory culture. These organisms are maintained in 10 gallon glass aquaria; plastic artificial turf and screen mesh serve as a substrate for the culture. The tanks are filled with de-chlorinated Lake Michigan water (City of Traverse City, Michigan water passed through an activated carbon filter). Cultures are fed 50 mL of 4 g/L Tetrafin slurry daily. When visible algae are not observed within the glass aquaria, algae (*Selenastrum* sp.) are used as a supplement to the Tetrafin slurry. Additionally, on occasion, dried Aspen (*Populus* sp.) leaves are prepared as a food supplement. The culture is maintained in a 16-hour light: 8-hour dark photoperiod at a temperature between 23 and 26 °C.

Test organisms were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 0.40, 0.67, 1.1, 1.8 and 3.0 mg NO₂-N/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ was determined using the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 44; test results are provided in Table 45. Analytical chemistry data are provided in Table 46. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 13.

Table 44. Test conditions for 96-hour toxicity test on *Hyalella azteca* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Hyalella azteca</i> , 10 days old, GLEC culture
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	August 6-August 10, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	100 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg NO ₂ -N/L):	3.0, 1.8, 1.1, 0.67 and 0.40
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg NO ₂ -N/L):	4.1, 2.4, 1.4, 0.81 and 0.54
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Nitrite: Sigma Aldrich, 97 +%, ACS Reagent Cas. No. 7632-00-0, Batch # 08316DJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 45. Test results for 96-hour toxicity test on *Hyalella azteca* with nitrite.

Results of a <i>Hyalella azteca</i> 96-Hour Static Acute Toxicity Test Conducted 08/06/09 - 08/10/09 Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	3.6	3.2	2.1	1.5
0.40 (0.54) mg/L	0 (0)	0 (0)	0 (0)	0 (0)	96-Hour LC₅₀* = 1.5 mg/L			
0.67 (0.81) mg/L	10 (10)	10 (10)	10 (10)	35 (35)	LC₅₀ 95% Confidence Limits			
1.1 (1.4) mg/L	10 (10)	15 (15)	30 (30)	50 (50)	24-Hr	48-Hr	72-Hr	96-Hr
1.8 (2.4) mg/L	30 (30)	40 (40)	60 (60)	70 (70)	LL 2.7	2.4	1.7	1.2
					UL 6.2	5.2	2.8	1.9
3.0 (4.1) mg/L	60 (65)	60 (65)	80 (80)	90 (90)	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 46. Analytical chemistry data for 96-hour toxicity test on *Hyaella azteca* with nitrite.

Nominal (and Measured) Test Concentrations		Nitrite ^a (mg NO ₂ -N/L)	Nitrate ^b (mg NO ₃ -N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.0011	<0.0012	22.0	8.00	8.4	300	60	80	ND
	Day 1			22.1	7.99	8.5				
	Day 2			23.2	7.77	8.3				
	Day 3			22.8	7.96	8.5				
	Day 4	0.02	0.044	22.1	7.80	8.0	290	60	80	
		0.02	0.056							
		0.02								
0.40 (0.54) mg/L	Day 0	0.51	<0.0048	22.0	7.98	8.4	336			
	Day 1			22.2	7.97	8.5				
	Day 2			23.2	7.99	8.4				
	Day 3			22.8	7.98	8.5				
	Day 4	0.57	<0.0001	22.1	7.91	8.0	310			
		0.54								
0.67 (0.81) mg/L	Day 0	0.87	<0.0072	22.0	7.99	8.4	340			
			<0.0072							
	Day 1			22.2	7.92	8.5				
	Day 2			23.1	8.01	8.4				
	Day 3			22.7	8.04	8.5				
	Day 4	0.78	0.055	22.1	7.90	8.0	315			
		0.79								
		0.81								
1.1 (1.4) mg/L	Day 0	1.44	<0.01	22.0	8.00	8.4	340			
	Day 1			22.2	7.95	8.5				
	Day 2			23.1	8.06	8.5				
	Day 3			22.8	8.06	8.6				
	Day 4	1.43	<0.01	22.2	7.90	8.0	325			
		1.44								
1.8 (2.4) mg/L	Day 0	2.40	<0.02	22.0	8.04	8.4	341			
	Day 1			22.2	7.90	8.5				
	Day 2			23.1	8.06	8.5				
	Day 3			22.8	8.04	8.6				
	Day 4	2.47	<0.02	22.2	7.96	8.0	325			
		2.44								
3.0 (4.1) mg/L	Day 0	3.96	<0.05	22.0	8.06	8.4	342	60	80	ND
	Day 1			22.2	7.90	8.5				
	Day 2			23.1	8.07	8.6				
	Day 3			22.8	8.04	8.5				
	Day 4	4.29	<0.05	22.2	7.97	8.0	322	58	80	
		4.1								

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM 4500 NO₃

ND Not Detect; below detection limit

96-hr Toxicity of Nitrite on *Pimephales promelas*

The 96-hr test to determine the toxicity of nitrite on *P. promelas* was completed by GLEC. Test organisms, collected from the GLEC laboratory culture, were acclimated to the dilution water (de-chlorinated Lake Michigan water), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); two replicates were used per treatment with ten organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 3.9, 6.6, 11, 18 and 30 mg NO₂-N/L.

Testing was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Trimmed Spearman-Kärber and Probit methods.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 47; test results are provided in Table 48. Analytical chemistry data are provided in Table 49. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 14.

Table 47. Test conditions for 96-hour toxicity test on *Pimephales promelas* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (weight 0.12g and 18 mm length), GLEC culture
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	August 13-August 17, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	4000 mL beaker
9. Volume of Test Solutions:	3500 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg NO ₂ -N/L):	30, 18, 11, 6.6, and 3.9
14. Measured Test Concentrations (average of samples collected at test initiation and termination- mg NO ₂ -N/L):	33, 18, 11, 5.8, and 4.1
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Sodium Nitrite: Sigma Aldrich, 97 +%, ACS Reagent Cas. No. 7632-00-0, Batch # 08316DJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 48. Test results for 96-hour toxicity test on *Pimephales promelas* with nitrite.

Results of a <i>Pimephales promelas</i> 96-Hour Static Acute Toxicity Test Conducted 08/13/09 - 08/17/09 Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	5 (5)	5 (5)	5 (5)	5 (5)	>33	>33	>33	27.0
3.9 (4.1) mg/L	10 (10)	10 (10)	10 (10)	15 (15)	96-Hour LC ₅₀ * = 27.0 mg/L			
6.6 (5.8) mg/L	10 (10)	10 (10)	10 (10)	10 (10)				
					LC ₅₀ 95% Confidence Limits			
11 (11) mg/L	0 (0)	0 (0)	0 (0)	5 (15)	24-Hr	48-Hr	72-Hr	96-Hr
18 (18) mg/L	20 (20)	25 (25)	30 (30)	35 (45)	LL NA	NA	NA	19.7
					UL NA	NA	NA	41.4
30 (33) mg/L	10 (25)	25 (25)	45 (65)	65 (65)				
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit: Trimmed Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 49. Analytical chemistry data for 96-hour toxicity test on *Pimephales promelas* with nitrite.

Nominal (Measured) Test Concentration		Nitrite ^a (mg NO2-N/L)	Nitrate ^b (mg NO3-N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)		Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.0011	0.23	25.0	7.99	8.1	8.2	290	102	144	ND
			0.23								
	Day 1			24.6	7.66	6.7	6.7				
	Day 2			24.2	7.55	6.1	5.8				
	Day 3			24.5	7.59	6.0					
	Day 4	0.01	0.26	24.7	7.67	6.0	6.1	304	100	136	ND
		1.15			7.69						
		0.01									
3.9 (4.1) mg/L	Day 0	3.90	<0.11	25.0	8.06	8.2	8.2	320			
	Day 1			24.6	7.90	7.1	6.9				
	Day 2			24.2	7.60	5.9	5.9				
	Day 3			24.4	7.65	6.0					
	Day 4	4.20	<0.11	24.7	7.74	5.9	5.9	333			
		4.26			7.77						
		4.12									
6.6 (5.8) mg/L	Day 0	5.18	0.52	25.0	8.07	8.4	8.3	330			
	Day 1			24.4	7.79	6.9	6.9				
	Day 2			24.3	7.56	5.5	5.9				
	Day 3			24.6	7.69	6.1					
	Day 4	6.39	<0.11	24.7	7.80	5.9	6.0	354			
		5.78			7.80						
11 (11) mg/L	Day 0	10.65	<0.25	25.0	8.08	8.5	8.3	360			
	Day 1			24.3	7.79	7.3	7.2				
	Day 2			24.2	7.63	6.5	6.1				
	Day 3			24.6	7.74	6.3					
	Day 4	10.65	<0.11	24.7	7.80	5.7	5.9	386			
		10.65			7.81						
18 (18) mg/L	Day 0	17.96	0.34	25.0	8.08	8.5	8.4	424			
	Day 1			24.3	7.79	7.3	7.3				
	Day 2			24.3	7.63	6.0	6.1				
	Day 3			24.3	7.69	6.0					
	Day 4	17.35	4.88	24.6	7.75	5.9	6.1	437			
					7.68						
		17.66									
30 (33) mg/L	Day 0	31.66	<0.61	25.0	8.06	8.6	8.5	507	98	136	ND
	Day 1			24.3	7.77	7.5	7.4				
	Day 2			24.2	7.61	6.3	6.5				
	Day 3			24.3	7.63	5.5					
	Day 4	33.79	<0.36	24.5	7.75	5.7	5.7	520	100	132	ND
		32.72			7.77						

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM4500 NO₃

ND Not Detect; below detection limit

32-day Toxicity of Nitrite on *Pimephales promelas*

The 32-day test to determine the toxicity of nitrite on *P. promelas* was completed by GLEC. The fish were continuously exposed for 32 days to five concentrations of nitrite (nominal concentrations of 1.9, 3.8, 7.6, 15 and 30 mg NO₂-N/L – dilution factor of 0.5) and to a dilution water control using a continuous flow-through system (Benoit et al. 1982). The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were four replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with < 24 hour old fertilized embryos. The embryos were randomly assigned to incubation cups until each incubation cup contained 30 embryos. The incubation cups were randomly assigned to the 2.5 L glass test chambers (1 cup per chamber) and suspended in the test solutions from a rocker arm assembly. The rocker arm assembly moves the incubation cups in a reciprocal motion within each test chamber. Embryos were inspected on a daily basis and the number of live, hatched and dead embryos was recorded. On Day 5 of the test (three days after first hatch), the surviving fish were randomly thinned to achieve 20 fish in each test chamber. The remainder of the surviving fish was discarded. The number of surviving fish was recorded at test termination (32 days). In addition, the wet weights were recorded for each fish at test termination. Because of the size range of fish in each test chamber, all of the fish from each test chamber were weighed together to determine average dry weight.

Instantaneous water temperature measurements made on August 11, 2009 (Day 4) ranged from 25.5°C -26.3°C within individual replicates; this exceeded the allowed range of 25 ± 1 °C in the toxicity testing method. However, the average water temperature (across the duration of the test) in each replicate was always ± 0.3 °C of the target test temperature (25 °C) in all treatments. In addition, the water temperature as recorded by the continuous temperature logger was never outside the range outlined in the method. Therefore, the water temperature exceedance likely had no effect on the results of this test.

In addition, on test day 24 there was an instance of low DO (5.8 mg/L) in replicate four of the laboratory control. Low DO concentrations were never observed on any other day of the test. Therefore, the one instance of low DO was an anomaly and likely had no effect on the results of this test.

On August 27, 2009 (day 20) a diluter malfunction (stock solution metering pump) occurred for 8 hours. Analytical chemistry samples were collected from replicate one of each test concentration subsequent to this malfunction that analysis indicated that the test solution concentrations were significantly less than the target concentrations. The measured concentration of nitrite in the five test concentrations on day 20 was less than 50 percent of the time-weighted average measured concentration; the duration of the malfunction was less than 10 percent (approximately 3.1 percent) of the total duration of

the test. Therefore, the diluter malfunction likely had little, if any, affect on the results of this test.

Once the test was complete, the LC₅₀, NOEC and LOEC values were determined using the average measured concentrations with the Spearman Karber and ANOVA methods while LC₂₅, LC₂₀, LC₁₀ values were determined with the Probit method. EC₅₀, EC₂₅, EC₂₀ and EC₁₀ values were estimated using EPA's TRAP.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 50; test results are provided in Table 51. Survival and growth data are provided in Table 52 and analytical chemistry data are provided in Table 53. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 15.

Table 50. Test conditions for 32-day toxicity test on *Pimephales promelas* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (<24 hour fertilized embryos), GLEC culture
2. Test Type and Duration:	Continuous flow-through; 32 days
3. Test Dates:	August 7-September 8, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	Live Brine Shrimp (<i>Artemia nauplii</i>) Twice daily
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume of Test Solutions:	2000 mL
10. No. of Test Organisms per Test Vessel:	30 eggs, thinned to 20 larvae after hatch
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	120 eggs, thinned to 80 larvae after hatch
13. Target or Nominal Test Concentrations (mg NO ₂ -N/L):	30, 15, 7.6, 3.8, and 1.9
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg NO ₂ -N/L):	30, 15, 6.8, 3.4, and 1.7
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Sodium Nitrite: Sigma Aldrich, 97 +%, ACS Reagent Cas. No. 7632-00-0, Batch # 08316DJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Survival (LC ₅₀ , LC ₂₅ , LC ₂₀ and LC ₁₀ , NOEC and LOEC) and Growth (EC ₅₀ , EC ₂₅ , EC ₂₀ and EC ₁₀ , NOEC and LOEC)

Table 51. Test results for 32-day toxicity test on *Pimephales promelas* with nitrite.

Results of a <i>Pimephales promelas</i> 32-Day Continuous Flow Chronic Toxicity Test						
Conducted 08/03/09 - 09/08/09 Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)						
Test Solution Concentrations Measured	Primary Control/ Dilution Water Secondary	1.7 mg/L	3.4 mg/L	6.8 mg/L	15 mg/L	30 mg/L
Embryo Percent Hatch (%)	100	97.5	95.8	97.5	96.7	99.2
32-Day Mean Survival (%)	90.0	88.8	86.3	78.8	38.8 ^a	0 ^a
32-Day Average Biomass ¹ (mg)	6.02	6.11	6.49	6.17	2.95 ^a	0 ^a
Embryo Percent Hatch NOEC:	30.2 mg/L		32-Day LC ₂₀ :	5.7 mg/L (4.3 mg/L – 7.0 mg/L)		
32-Day LC ₅₀ *:	11.3 mg/L (9.7 mg/L-13.2 mg/L)		32-Day LC ₁₀ :	2.3 mg/L (0.6 mg/L – 4.0 mg/L)		
32-Day Survival NOEC:	6.8 mg/L		32-Day EC ₅₀ :	14.6 mg/L (12.7 mg/L – 16.8 mg/L)		
32-Day Survival LOEC:	15.0 mg/L		32-Day EC ₂₅ :	11.7 mg/L (6.7 mg/L – 20.4 mg/L)		
32-Day Growth NOEC:	6.8 mg/L		32-Day EC ₂₀ :	11.0 mg/L (5.6 mg/L – 21.8 mg/L)		
32-Day Growth LOEC:	15.0 mg/L		32-Day EC ₁₀ :	9.3 mg/L (3.3 mg/L – 26.1 mg/L)		
32-Day LC ₂₅ :	7.0 mg/L (5.7 mg/L – 8.2 mg/L)					

^a: Significantly different from dilution water control ($p \leq 0.05$)

*: All LC, EC, NOEC and LOEC values are determined based on the average measured nitrite concentration.

NOEC: No-Observed-Effect-Concentration

LOEC: Lowest-Observed-Effect-Concentration

¹Biomass: Biomass is the average dry weight of the four replicates calculated by the total dry weight of surviving organisms divided by the initial number of organisms (20).

Table 52. Survival and growth data for 32-day toxicity test on *Pimephales promelas* with nitrite.

Nominal (and Measured) Test Concentration		Number of Eggs at Test Initiation	Number of Hatched Larvae	Number of Dead Eggs	Percent Hatched Larvae	Number of Larvae at Test Termination	Percent Survival at Test Termination*	Biomass ¹ (mg)
Dilution water/Control	Replicate # 1	30	30	0	100.0	20	100.0	5.72
	Replicate # 2	30	30	0	100.0	19	95.0	6.28
	Replicate # 3	30	30	0	100.0	17	85.0	5.73
	Replicate # 4	30	30	0	100.0	16	80.0	6.33
	Average				100.0		90.0	6.02
1.9 (1.7) mg/L	Replicate # 1	30	29	1	96.7	16	80.0	6.86
	Replicate # 2	30	30	0	100.0	17	85.0	5.69
	Replicate # 3	30	30	0	100.0	18	90.0	5.77
	Replicate # 4	30	28	2	93.3	20	100.0	6.12
	Average				97.5		88.8	6.11
3.8 (3.4) mg/L	Replicate # 1	30	29	1	96.7	19	95.0	6.37
	Replicate # 2	30	26	4	86.7	12	60.0	7.04
	Replicate # 3	30	30	0	100.0	19	95.0	6.26
	Replicate # 4	30	30	0	100.0	19	95.0	6.28
	Average				95.8		86.3	6.49
7.6 (6.8) mg/L	Replicate # 1	30	28	2	93.3	16	80.0	6.55
	Replicate # 2	30	29	1	96.7	16	80.0	6.28
	Replicate # 3	30	30	0	100.0	19	95.0	5.51
	Replicate # 4	30	30	0	100.0	12	60.0	6.34
	Average				97.5		78.8	6.17
15 (15) mg/L	Replicate # 1	30	30	0	100.0	9	45.0	3.27
	Replicate # 2	30	28	2	93.3	2	10.0	0.88
	Replicate # 3	30	29	1	96.7	9	45.0	3.43
	Replicate # 4	30	29	1	96.7	11	55.0	4.22
	Average				96.7		38.8	2.95
30 (30) mg/L	Replicate # 1	30	30	0	100.0	0	0.0	0.00
	Replicate # 2	30	29	1	96.7	0	0.0	0.00
	Replicate # 3	30	30	0	100.0	0	0.0	0.00
	Replicate # 4	30	30	0	100.0	0	0.0	0.00
	Average				99.2		0.0	0.00

* On Day 5 of the test, (three days after first hatch) the surviving fish were randomly thinned to 20 fish in each test chamber. Percent survival at test termination is the number of surviving at test termination divided by 20.

¹ Biomass: Biomass is the total dry weight of surviving organisms divided by the initial number of organisms (20)

Table 53. Analytical chemistry data for 32-day toxicity test on *Pimephales promelas* with nitrite.

Nominal (and Measured) Test Concentrations		NO ₂ (mg NO ₂ -N/L)	NO ₃ (mg NO ₃ -N/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmhos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate # 1	0.00 (0.00-0.01)	0.25	24.8 (24.0-25.4)	8.03	7.7 (6.9-8.5)	298	118	105	ND
	Replicate # 2	0.01 (0.01-0.01)	0.26	25.2 (24.3-26.3)	8.13	8.1 (7.7-9.1)	304			ND
	Replicate # 3	0.00 (0.00-0.01)	0.26	25.2 (24.4-25.7)	8.17	8.0 (7.4-9.4)				ND
	Replicate # 4	0.01 (0.00-0.03)	0.25	25.3 (24.2-26.2)	8.03	7.3 (5.8-8.2)	301	132	102	ND
	Average	0.01								
1.9 mg/L (1.7 mg/L)	Replicate # 1	1.44 (0.70*-2.04)	0.22	24.9 (24.1-25.5)	8.18	7.8 (6.7-9.1)	315	146	103	ND
	Replicate # 2	1.60 (0.88-1.95)	0.26	25.2 (24.0-26.0)	8.25	8.0 (7.7-8.3)	324			ND
	Replicate # 3	1.99 (1.52-2.41)	0.11	25.1 (24.5-25.8)	8.17	7.9 (7.7-8.0)				ND
	Replicate # 4	1.86 (1.46-2.47)	0.25	25.1 (24.1-26.0)	8.23	7.8 (6.7-9.1)	318	132	106	ND
	Average	1.7								
3.8 mg/L (3.4 mg/L)	Replicate # 1	3.07 (1.40*-4.20)	0.26	24.9 (24.0-25.6)	8.22	7.9 (7.0-9.2)	328	140	105	ND
	Replicate # 2	3.5 (3.01-4.47)	0.22	25.0 (24.1-26.1)	8.27	8.1 (7.7-9.2)	292			ND
	Replicate # 3	3.65 (2.92-4.29)	0.32	25.1 (24.5-25.9)	8.10	7.8 (7.5-8.1)				ND
	Replicate # 4	3.56 (3.04-4.17)	0.14	25.1 (24.2-25.9)	8.18	7.7 (6.9-8.2)	326	132	104	ND
	Average	3.4								
7.6 mg/L (6.8 mg/L)	Replicate # 1	6.36 (2.83*-7.34)	0.41	25.1 (24.1-25.8)	8.18	7.6 (7.0-8.0)	359	136	105	ND
	Replicate # 2	6.91 (5.78-7.85)	0.44	25.2 (24.2-25.9)	8.26	7.9 (7.3-9.1)	368			ND
	Replicate # 3	7.06 (6.09-8.22)	0.28	25.1 (24.3-25.7)	8.18	7.8 (7.4-8.0)				ND
	Replicate # 4	7.31 (6.09-8.13)	0.17	25.0 (24.3-26.1)	8.23	7.7 (6.4-9.1)	357	132	106	ND
	Average	6.8								
15 mg/L (15 mg/L)	Replicate # 1	14.1 (4.63*-17.5)	0.81	25.0 (24.0-26.0)	8.21	7.9 (7.2-9.1)	417	144	104	ND
	Replicate # 2	15.0 (12.8-18.5)	0.76	25.0 (24.2-25.8)	8.27	7.9 (7.6-8.3)	431			ND
	Replicate # 3	15.9 (15.0-17.2)	1.17	25.0 (24.3-25.7)	8.19	7.7 (7.6-8.0)				ND
	Replicate # 4	15.3 (14.3-16.6)	0.61	25.3 (24.5-26.1)	8.29	8.0 (7.0-9.2)	426	124	104	ND
	Average	15								
30 mg/L (30 mg/L)	Replicate # 1	30.0 (10.7*-51.8)	3.16	24.6 (24.0-25.4)	8.24	8.0 (7.4-9.4)	528	136	103	ND
	Replicate # 2	30.4 (25.0-34.4)	1.75	25.0 (24.3-26.3)	8.28	8.1 (7.5-9.0)	545			ND
	Replicate # 3	29.7 (21.0-42.3)	4.74	25.0 (24.3-25.8)	8.25	8.1 (8.0-8.2)				ND
	Replicate # 4	30.7 (28.6-32.6)	1.81	25.1 (24.5-26.2)	8.29	7.7 (7.1-8.3)	454	132	104	ND
	Average	30								

DO: Dissolved Oxygen

SC: Specific Conductance

ND: Non Detect; below detection limit.

* August 20,2009 (day 20) a metering diluter malfunction occurred and analytical chemistry samples were collected.

48-hr Toxicity of Nitrite on *Ceriodaphnia dubia*

The 48-hr test to determine the toxicity of nitrite on *C. dubia* was completed by GLEC. Test organisms, < 24-hours old collected from the GLEC laboratory culture, were acclimated to the dilution water (DMW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 0.99, 1.6, 2.7, 4.6, and 7.6 mg NO₂-N/L.

Testing was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Probit and Spearman Karber methods.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 54; test results are provided in Table 55. Analytical chemistry data are provided in Table 56. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 16.

Table 54. Test conditions for 48-hour toxicity test on *Ceriodaphnia dubia* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Ceriodaphnia dubia</i> , < 24 hours old, GLEC culture
2. Test Type and Duration:	Static, 48 hours
3. Test Dates:	August 21-August 23, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	30 mL beaker
9. Volume Test Solutions:	15 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (NO ₂ -N/L):	7.6, 4.6, 2.7, 1.6, and 0.99
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- NO ₂ -N/L):	7.3, 4.7, 2.8, 1.6, and 0.99
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA DMW
17. Test Material:	Sodium Nitrite: Sigma Aldrich, 97 +%, ACS Reagent Cas. No. 7632-00-0, Batch # 08316DJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 55. Test results for 48-hour toxicity test on *Ceriodaphnia dubia* with nitrite.

Results of a <i>Ceriodaphnia dubia</i> 48-Hour Static Acute Toxicity Test Conducted 08/21/09 - 08/23/09 Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)			5.8	4.6	NA	NA
1.0 (1.0) mg/L	0 (0)	0 (0)			48-Hour LC₅₀* = 4.6 mg/L			
1.6 (1.6) mg/L	0 (0)	0 (0)			LC₅₀* 95% Confidence Limits			
2.7 (2.8) mg/L	0 (0)	0 (0)			24-Hr	48-Hr	72-Hr	96-Hr
4.6 (4.7) mg/L	25 (25)	50 (50)			LL 5.1	4.1	NA	NA
					UL 6.6	5.1	NA	NA
7.6 (7.3) mg/L	80 (80)	100 (100)			LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Spearman-Kärber and Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 56. Analytical chemistry data for 48-hour toxicity test on *Ceriodaphnia dubia* with nitrite.

Nominal (and Measured) Test Concentration		Nitrite ^a (mg NO ₂ -N/L)	Nitrate ^b (mg NO ₃ -N/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.0011	0.31	25.0	7.98	7.8	189	76	96	ND
		<0.0011	0.30							
	Day 1			24.6	8.40	8.8				
	Day 2	0.020	0.17	24.8	8.08	8.9	192			
Average		0.020								
1.0 (1.0) mg/L	Day 0	0.99	0.12	25.0	8.10	7.8	206			
	Day 1			24.7	8.32	8.8				
	Day 2	0.98	0.33	24.8	8.12	9.0	210			
	Average	0.99								
1.6 (1.6) mg/L	Day 0	1.72	0.26	25.0	8.11	7.9	214			
	Day 1			24.7	8.24	8.8				
	Day 2	1.52	0.31	24.8	8.20	9.0	220			
	Average	1.63								
2.7 (2.8) mg/L	Day 0	2.98	0.05	25.0	8.12	8.0	223			
	Day 1			24.6	8.24	8.8				
	Day 2	2.62	0.37	24.8	8.22	9.0	229			
	Average	2.80								
4.6 (4.7) mg/L	Day 0	4.7	<0.18	25.0	8.13	8.0	236			
	Day 1			24.6	8.22	8.9				
	Day 2	4.7	0.41	24.8	8.30	9.0	234			
	Average	4.7								
7.6 (7.3) mg/L	Day 0	7.6	<0.11	25.0	8.14	8.0	257	76	108	ND
	Day 1			24.6	8.21	9.0				
	Day 2	7.0	<0.11	24.8	8.36	9.0	246			
	Average	7.3								

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM4500 NO₃

ND Not Detect; below detection limit

7-day Toxicity of Nitrite on *Ceriodaphnia dubia*

The 7-day test to determine the toxicity of nitrite on *C. dubia* was completed by GLEC. Test organisms, < 24-hours old collected from the GLEC laboratory culture, were acclimated to the dilution water (DMW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); ten replicates were used per treatment with one organism per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static-renewal conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 0.48, 0.95, 1.9, 3.8, and 7.6 mg NO₂-N/L.

Testing was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). On a daily basis, organisms were fed *Selenastrum capricornutum* and YTC (at renewal) and examined for mortality. As outlined in ASTM Method E1295 (ASTM 2006 b), the duration of this test is based primarily on brood production. Therefore, because greater than 60% of surviving control females had three broods at Day 6 of the test, the test was terminated early and no results were reported for Day 7.

Once the test was complete, the 48-hour and 6-day LC₅₀ values and 6-day NOEC and LOEC values were determined using the average measured concentrations with the Spearman Karber and ANOVA methods. LC₂₅, LC₂₀ and LC₁₀ values were determined using the Probit method and EC₅₀, EC₂₅, EC₂₀ and EC₁₀ values were estimated using EPA's TRAP.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 57; test results are provided in Table 58. Analytical chemistry data are provided in Table 59. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 17.

Table 57. Test conditions for 7-day toxicity test on *Ceriodaphnia dubia* with nitrite.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Ceriodaphnia dubia</i> , < 24 hours old, GLEC culture
2. Test Type and Duration:	Three Brood Static-Renewal
3. Test Dates:	August 21-August 27, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	Daily: <i>Selenastrum capricornutum</i> and YTC at renewal
8. Size of Test Vessel:	30 mL beaker
9. Volume Test Solutions:	15 mL
10. No. of Test Organisms per Test Vessel:	1
11. No. of Test Vessels per Treatment:	10
12. Total No. of Test Organisms per Treatment:	10
13. Target or Nominal Test Concentrations (mg NO ₂ -N/L):	7.6, 3.8, 1.9, 0.95, and 0.48
14. Analytical Test Concentrations (average of samples collected at test initiation and termination- mg NO ₂ -N/L):	7.3, 3.6, 1.8, 0.82, and 0.36
15. Renewal of Test Solutions:	Daily
16. Dilution and Primary Control Water:	USEPA DMW
17. Test Material:	Sodium Nitrite: Sigma Aldrich, 97 +%, ACS Reagent Cas. No. 7632-00-0, Batch # 08316DJ
18. Secondary Control Water:	Boardman River Culture Water
19. Aeration:	None
20. Endpoints Measured:	Survival (LC ₅₀ , LC ₂₅ , LC ₂₀ and LC ₁₀ , NOEC and LOEC) and Reproduction (EC ₅₀ , EC ₂₅ , EC ₂₀ and EC ₁₀ , NOEC and LOEC)

Table 58. Test results for 7-day toxicity test on *Ceriodaphnia dubia* with nitrite.

Results of a <u><i>Ceriodaphnia dubia</i></u> 7-Day Static Renewal Chronic Toxicity Test Conducted <u>08/21/09 - 08/27/09</u> Using: Nitrite (Sodium Nitrite: Sigma Aldrich Cas. No. 7632-00-0)							
Nominal (Measured) Concentrations	Primary Control/ Dilution Water	Secondary Control	0.48 (0.36) mg/L	0.95 (0.82) mg/L	1.90 (1.75) mg/L	3.81 (3.56) mg/L	7.61 (7.31) mg/L
48-hour Survival (%)	100	100	100	100	100	100	0
6-day Mean Survival (%)	100	100	100	90	100	90	0
6-day Mean Reproduction/Female	25.1	20.7	25.0	23.3	19.1	3.6	0
48-Hour LC ₅₀ *	5.1 mg/L		6-Day LC ₂₀ :		3.3 mg/L (2.3 mg/L – 4.3 mg/L)		
6-Day LC ₅₀ *	4.7 mg/L (4.1 mg/L - 5.4 mg/L)		6-Day LC ₁₀ :		2.5 mg/L (1.4 mg/L – 3.6 mg/L)		
6-Day Mean Survival (%) NOEC*	3.6 mg/L		6-Day EC ₅₀ :		2.4 mg/L (2.1 mg/L – 2.8 mg/L)		
6-Day Mean Survival (%) LOEC*	7.3 mg/L		6-Day EC ₂₅ :		1.9 mg/L (1.5 mg/L – 2.3 mg/L)		
6-Day Mean Reproduction per Female NOEC*	0.82 mg/L		6-Day EC ₂₀ :		1.7 mg/L (1.4 mg/L – 2.2 mg/L)		
6-Day Mean Reproduction per Female LOEC*	1.8 mg/L		6-Day EC ₁₀ :		1.4 mg/L (1.1 mg/L – 1.9 mg/L)		
6-Day LC ₂₅ :	3.5 mg/L (2.5 mg/L – 4.6 mg/L)						

* All LC, EC, NOEC and LOEC values are determined based on the analytical chemistry results.

NOEC: No Observed Effect Concentration

LOEC: Lowest Observed Effect Concentration

Table 59. Analytical chemistry data for 7-day toxicity test on *Ceriodaphnia dubia* with nitrite.

Nominal (and Measured) Test Concentration		Nitrite ^a (mg NO ₂ -N/L)	Nitrate ^b (mg NO ₃ -N/L)	Temperature (°C) old	pH (s.u.) new ^N old ^D		D.O. (mg/L) new ^N old ^D		Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0 ^N	<0.0011	0.307	24.7	8.00		7.9		183	76	96	ND
		<0.0011	0.305									
	Day 1 ^O	0.006	<0.0012	24.7	8.16	8.35	8.0	8.7	195			
		0.005	<0.0012									
	Day 2 ^N	0.002	0.258	24.7	8.20	8.47	8.0	9.0	180			
	Day 3 ^O			24.6	8.21	8.88	7.3	8.9	197	74	100	
	Day 4 ^N	0.02	0.061	25.1	8.10	8.56	8.2	8.9	187			
	Day 5 ^O	0.01	0.079	24.7	8.12	8.47	7.6	9.0	181	58	88	ND
Average			0.074									
		0.02	<0.0012			8.31		8.9				
			<0.0012									
0.48 (0.366) mg/L	Day 0 ^N	0.50	0.111		8.12		7.9		196			
	Day 1 ^O	0.34	0.184		8.19	8.37	8.0	8.7	210			
	Day 2 ^N	0.48	0.167		8.22	8.49	8.0	9.0	203			
	Day 3 ^O	0.23	0.111		8.28	8.87	7.3	8.9	204			
	Day 4 ^N				8.03	8.60	8.2	8.9	191			
	Day 5 ^O				8.08	8.67	8.0	9.0	188			
	Day 6 ^O	0.26	0.159			8.43		8.9				
	Average		0.36									
0.95 (0.82) mg/L	Day 0 ^N	1.00	0.239		8.16		8.0		206			
	Day 1 ^O	0.80	0.255		8.20	8.37	8.0	8.7	217			
	Day 2 ^N				8.23	8.50	8.0	9.0	218			
	Day 3 ^O				8.29	8.87	7.3	8.9	208			
	Day 4 ^N	0.85	0.357		8.05	8.64	8.2	8.9	199			
	Day 5 ^O	0.74	0.172		8.10	8.64	7.8	9.3	194			
	Day 6 ^O	0.72	0.251			8.44		8.9				
	Average		0.82									
1.90 (1.75) mg/L	Day 0 ^N	1.94	0.151	24.7	8.15		8.0		213			
	Day 1 ^O	1.61	0.233	24.8	8.19	8.39	8.0	8.7	225			
	Day 2 ^N	1.98	0.323	24.4	8.23	8.54	8.0	9.0	231			
	Day 3 ^O	1.70	0.145	24.6	8.29	8.87	7.4	8.9	215			
		1.61	0.215									
	Day 4 ^N			25.2	8.07	8.61	8.2	8.9	206			
	Day 5 ^O			24.7	8.09	8.66	7.9	9.0	201			
	Day 6 ^O	1.67	0.124			8.39		8.9				
	Average		1.75									
3.81 (3.56) mg/L	Day 0 ^N	3.77	0.291		8.16		8.1		227			
	Day 1 ^O	3.81	0.041		8.15	8.40	8.0	8.7	238	76	104	
	Day 2 ^N				8.23	8.54	8.0	9.0	244	76	108	
	Day 3 ^O				8.27	8.84	7.2	8.9	229	74	104	
	Day 4 ^N	3.62	0.558		8.08	8.67	8.2	8.9	222	76	108	
	Day 5 ^O	3.29	0.818		8.09	8.67	7.9	9.1	215	76	108	ND
	Day 6 ^O	3.38	0.332			8.43		8.9				
	Average		3.57									
7.61 (7.31) mg/L	Day 0 ^N	7.6	<0.11		8.14		8.4		253	76	108	ND
	Day 1 ^O	7.0	<0.11		8.17	8.40	8.5	8.7	261	76	104	
	Day 2 ^N											
	Day 3 ^O											
	Day 4 ^N											
	Day 5 ^O											
	Day 6 ^O											
	Average		7.3									

^a Nitrite Analysis Method SM 4500 NO₂

^b Nitrate Analysis Method SM 4500 NO₃

ND Not Detect; below detection limit

^N "New" test solutions are fresh solutions prepared each day and are used to initiate the toxicity test and to renew the existing test solutions.

Boron

Table 60 provides a summary of estimated LC₅₀ values for the nine toxicity tests performed using boron. LC₅₀ values ranged between 28.4 and >544 mg B/L.

Table 60. LC₅₀ estimates for toxicity tests performed using boron.

Test Species and Duration	LC ₅₀ (mg B/L)
<i>Lampsilis siliquioidea</i> - 96 hr	137
<i>Pimephales promelas</i> - 96 hr	101
<i>Pimephales promelas</i> - 32 day	28.4
<i>Ceriodaphnia dubia</i> - 48 hr (pH 7.75)	76.9
<i>Pimephales promelas</i> - 96 hr (pH 6.75)	70.6
<i>Pimephales promelas</i> - 96 hr (pH 7.75)	137
<i>Pimephales promelas</i> - 96 hr (pH 8.75)	133
<i>Ligumia recta</i> - 96 hr	147
<i>Megaloniaias nervosa</i> - 96 hr	> 544

For each of the acute toxicity tests completed using boron, two tables were generated: the first summarizes the test results for each toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity tests. The results of chronic tests performed with boron were summarized in three tables: the first summarizes nominal and analytical test concentrations, LC₅₀ estimates with confidence intervals, NOEC and LOEC estimates, mean survival and mean biomass; the second table summarizes replicate-specific survival and growth data and the third table summarizes analytical chemistry data collected throughout the toxicity tests. Also discussed, if applicable, are deviations from the guidance provided in the ASTM method used to complete the toxicity testing.

96-hr Toxicity of Boron on *Lampsilis siliquoidea*

The 96-hr test to determine the toxicity of boron on *L. siliquoidea* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Missouri State University laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 500, 250, 125, 62.5, and 31.3 mg B/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 61; test results are provided in Table 62. Analytical chemistry data are provided in Table 63. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 18.

Table 61. Test conditions for 96-hour toxicity test on *Lampsilis siliquoidea* with boron.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Lampsilis siliquoidea</i> , juveniles <5 days old (Missouri State University)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	June 03 - 07, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg B/L):	500, 250, 125, 62.5, and 31.3
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg B/L):	524, 260, 140, 72, and 34
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Boric acid: Acros Organics, 99.6%, ACS Reagent (crystals) Cas. No. 10043-35-3, Lot # B0124654 and Borax (sodium tetraborate decahydrate), 99.5 +% (for analysis ACS), Cas. No. 1303-96-5, Lot # A0256722
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 62. Test results for 96-hour toxicity test on *Lampsilis siliquoidea* with boron.

Results of a <i>Lampsilis siliquoidea</i> 96-Hour Static Acute Toxicity Test								
Conducted 06/03/09 - 06/07/09 Using: Boric acid Cas. No. 10043-35-3 and Borax Cas. No. 1303-96-5								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	5	5	5	5	>524	>524	181	137
31.3 (34) mg/L	0	10	25	35	96-Hour LC ₅₀ [*] = 137 mg/L			
62.5 (72) mg/L	0	25	30	35				
					LC ₅₀ 95% Confidence Limits			
125 (140) mg/L	10	15	35	45	24-Hr	48-Hr	72-Hr	96-Hr
250 (260) mg/L	15	25	60	90	LL NR	NR	110	86
					UL NR	NR	296	220
500 (524) mg/L	15	20	90	95	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 63. Analytical chemistry data for 96-hour toxicity test on *Lampsilis siliquoidea* with boron.

Nominal (Measured) Test Concentration		Boron ^a (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	0.1	20.0	8.0	7.81	305	60	90
	Day 1		19.9					
	Day 2		20.0					
	Day 3		20.1					
	Day 4	1.1	19.9	8.0	7.10	305	62	90
		0.3						
31.3 (34) mg/L	Day 0	35	20.1	8.0	7.80	322	82	90
	Day 1		19.8					
	Day 2		20.0					
	Day 3		20.1					
	Day 4	33	19.9	8.0	7.05	320	82	92
		34						
62.5 (72) mg/L	Day 0	69	20.0	8.0	7.80	344	90	90
	Day 1		19.8					
	Day 2		20.1					
	Day 3		20.1					
	Day 4	76	20.0	8.0	6.99	350	92	92
		72						
125 (140) mg/L	Day 0	130	20.2	8.0	7.92	385	116	90
	Day 1		19.7					
	Day 2		20.1					
	Day 3		20.1					
	Day 4	150	19.9	8.0	6.97	390	120	90
		140						
250 (260) mg/L	Day 0	250	20.0	8.0	7.96	464	164	88
	Day 1		19.9					
	Day 2		20.1					
	Day 3		20.1					
	Day 4	270	19.9	8.0	6.92	465	164	90
		260						
500 (524) mg/L	Day 0	500	20.1	8.1	7.99	619	272	86
	Day 1		20.0					
	Day 2		20.1					
	Day 3		20.1					
	Day 4	550	20.0	8.0	6.89	625	270	90
		524						

^a Boron Analysis Method 200.7

96-hr Toxicity of Boron on *Megalonaias nervosa*

The 96-hr test to determine the toxicity of boron on *M. nervosa* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Genoa National Fish Hatchery, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate. In one replicate of the 250 mg/L treatment, a test organism was inadvertently crushed, but this was accounted for in the LC₅₀ calculation.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 500, 250, 125, 62.5, and 31.3 mg B/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 64; test results are provided in Table 65. Analytical chemistry data are provided in Table 66. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 19.

Table 64. Test conditions for 96-hour toxicity test on *Megaloniaias nervosa* with boron.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Megaloniaias nervosa</i> , juveniles <5 days old, Genoa National Fish Hatchery
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	October 16 - 20, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg B/L):	500, 250, 125, 62.5, and 31.3
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg B/L):	544, 275, 140, 74, and 37
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Boric acid: Acros Organics, 99.6%, ACS Reagent (crystals) Cas. No. 10043-35-3, Lot # B0124654 and Borax (sodium tetraborate decahydrate), 99.5 +% (for analysis ACS), Cas. No. 1303-96-5, Lot # A0256722
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 65. Test results for 96-hour toxicity test on *Megaloniaias nervosa* with boron.

Results of a <i>Megaloniaias nervosa</i> 96-Hour Static Acute Toxicity Test Conducted 10/16/09 - 10/20/09 Using: Boric acid Cas. No. 10043-35-3 and Borax Cas. No. 1303-96-5								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	>544 >544 >544 >544 96-Hour LC ₅₀ * = >544mg/L			
31.3 (37) mg/L	0	0	0	0				
62.5 (74) mg/L	0	0	5	10	LC ₅₀ 95% Confidence Limits			
125 (140) mg/L	0	0	5	15	24-Hr	48-Hr	72-Hr	96-Hr
250 (275) mg/L	0	5	10	20	LL NR	NR	NR	NR
500 (544) mg/L	0	0	0	5	UL NR	NR	NR	NR
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 66. Analytical chemistry data for 96-hour toxicity test on *Megalonaias nervosa* with boron.

Nominal (Measured) Test Concentration		Boron ^a (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	<0.02	21.0	7.8	7.95	300	60	88
	Day 1		20.8	7.7	8.27	303		
	Day 2		20.5	7.8	8.21	305		
	Day 3		20.3	8.0	8.21	290		
	Day 4	<0.02	20.6	8.0	7.62	337	68	90
		na						
31.3 (37) mg/L	Day 0	36	21.0	8.0	8.21	320	70	88
	Day 1		20.5	7.9	8.32	320		
	Day 2		20.5	7.9	8.22	320		
	Day 3		20.4	8.0	8.28	330		
	Day 4	38	20.6	8.0	8.15	351	72	88
		37						
62.5 (74) mg/L	Day 0	72	21.0	8.0	8.20	340	86	88
	Day 1		20.8	7.9	8.34	343		
	Day 2		20.5	7.9	8.22	345		
	Day 3		20.3	8.0	8.27	347		
	Day 4	76	20.5	8.0	8.25	364	90	88
		74						
125 (140) mg/L	Day 0	140	21.0	7.9	8.25	381	110	88
	Day 1		20.9	8.0	8.38	389		
	Day 2		20.6	8.0	8.23	390		
	Day 3		20.5	8.1	8.29	401		
	Day 4	140	20.7	8.1	8.27	417	115	88
		140						
250 (275) mg/L	Day 0	270	21.0	7.9	8.23	460	160	88
	Day 1		20.8	8.0	8.40	461		
	Day 2		20.6	8.0	8.21	461		
	Day 3		20.5	8.1	8.25	488		
	Day 4	280	20.8	8.1	8.28	504	178	88
		275						
500 (544) mg/L	Day 0	520	21.0	7.9	8.24	613	266	86
	Day 1		20.9	8.0	8.32	616		
	Day 2		20.6	8.0	8.20	618		
	Day 3		20.4	8.1	8.25	638		
	Day 4	570	20.8	8.2	8.23	654	276	88
		544						

^a Boron Analysis Method 200.7

na=not applicable

96-hr Toxicity of Boron on *Ligumia recta*

The 96-hr test to determine the toxicity of boron on *L. recta* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Missouri State University laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate. One replicate was mistakenly loaded with only four individuals, but this was accounted for in the LC₅₀ calculation.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 500, 250, 125, 62.5, and 31.3 mg B/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 67; test results are provided in Table 68. Analytical chemistry data are provided in Table 69. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 20.

Table 67. Test conditions for 96-hour toxicity test on *Ligumia recta* with boron.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Ligumia recta</i> , juveniles <5 days old, Missouri State University
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 10 - 14, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg B/L):	500, 250, 125, 62.5, and 31.3
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg B/L):	510, 255, 130, 64, and 33
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Boric acid: Acros Organics, 99.6%, ACS Reagent (crystals) Cas. No. 10043-35-3, Lot # B0124654 and Borax (sodium tetraborate decahydrate), 99.5 +% (for analysis ACS), Cas. No. 1303-96-5, Lot # A0256722
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 68. Test results for 96-hour toxicity test on *Ligumia recta* with boron.

Results of a <i>Ligumia recta</i> 96-Hour Static Acute Toxicity Test Conducted 09/10/09 - 09/14/09 Using: Boric acid Cas. No. 10043-35-3 and Borax Cas. No. 1303-96-5								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	>510	>510	>510	147
31.3 (33) mg/L	0	0	0	30	96-Hour LC₅₀* = 147 mg/L			
62.5 (64) mg/L	0	0	0	30				
					LC ₅₀ 95% Confidence Limits			
125 (130) mg/L	0	0	0	50	24-Hr	48-Hr	72-Hr	96-Hr
250 (255) mg/L	0	0	0	58	LL NR	NR	NR	88
					UL NR	NR	NR	246
500 (510) mg/L	0	0	0	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 69. Analytical chemistry data for 96-hour toxicity test on *Ligumia recta* with boron.

Nominal (Measured) Test Concentration		Boron ^a (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	<0.02	20.4	7.9	8.18	301	60	92
	Day 1		19.5					
	Day 2		19.1					
	Day 3		19.2					
	Day 4	<0.02	19.2	8.1	8.14	312	60	92
		na						
31.3 (33) mg/L	Day 0	33	20.5	8.0	8.14	320	68	92
	Day 1		19.5					
	Day 2		19.0					
	Day 3		19.3					
	Day 4	34	19.3	8.1	8.10	334	68	92
		33						
62.5 (64) mg/L	Day 0	62	20.5	8.0	8.13	341	90	90
	Day 1		19.5					
	Day 2		19.0					
	Day 3		19.3					
	Day 4	66	19.3	8.1	8.11	353	90	90
		64						
125 (130) mg/L	Day 0	130	20.4	8.0	8.12	382	112	90
	Day 1		19.5					
	Day 2		19.0					
	Day 3		19.3					
	Day 4	130	19.3	8.1	8.05	394	112	90
		130						
250 (255) mg/L	Day 0	250	20.5	8.1	8.12	460	170	90
	Day 1		19.5					
	Day 2		19.2					
	Day 3		19.3					
	Day 4	260	19.3	8.1	8.03	472	170	90
		255						
500 (510) mg/L	Day 0	500	20.5	8.1	8.11	616	270	90
	Day 1		19.5					
	Day 2		19.4					
	Day 3		19.1					
	Day 4	520	19.1	8.1	8.12	634	270	90
		510						

^a Boron Analysis Method 200.7

na = not applicable

96-hr Toxicity of Boron on *Pimephales promelas*

The 96-hr test to determine the toxicity of boron on *P. promelas* was completed by GLEC. Test organisms, collected from the GLEC laboratory culture, were acclimated to the dilution water (de-chlorinated Lake Michigan water), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); two replicates were used per treatment with ten organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 38.9, 64.8, 108, 180, 300 and 500 mg B/L.

Testing was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 70; test results are provided in Table 71. Analytical chemistry data are provided in Table 72. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 21.

Table 70. Test conditions for 96-hour toxicity test on *Pimephales promelas* with boron.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (weight 0.12g and 19.8 mm length), GLEC Culture
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 2-September 6, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	4000 mL beaker
9. Volume of Test Solutions:	3500 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg B/L):	500, 300, 180, 108, 64.8, and 38.9
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg B/L):	546, 352, 200, 123, 71.5, and 46.1
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Boric Acid: Sigma Aldrich, ACS Reagent ≥ 99.5% Cas. No. 10043-35-3, Batch 118K0007 and Borax (sodium tetraborate decahydrate) Sigma Aldrich, ≥ 99.5%, ACS reagent, Cas. No. 1303-96-4, Lot # 118K0172
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 71. Test results for 96-hour toxicity test on *Pimepehales promelas* with boron.

Results of a <i>Pimepehales promelas</i> 96-Hour Static Acute Toxicity Test								
Conducted <u>09/02/09 - 09/06/09</u> Using: Boron (Boric Acid: Sigma Aldrich Cas No. 10043 35-3) (Borax: Sigma Aldrich Cas No. 1303-96-4)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ * Values (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	>546	312	173	101
38.9 (46.1) mg/L	0 (0)	0 (0)	0 (0)	0 (0)	96-Hour LC ₅₀ *= 101 mg/L			
64.8 (71.5) mg/L	0 (0)	0 (0)	0 (5)	15 (15)	LC ₅₀ *95% Confidence Limits			
108 (123) mg/L	0 (0)	0 (0)	20 (20)	70 (70)	24-Hr	48-Hr	72-Hr	96-Hr
180 (200) mg/L	0 (0)	5 (5)	60 (60)	100 (100)	LL NA	271	150	88.3
					UL NA	353	200	116
300 (352) mg/L	0 (0)	65 (65)	100 (100)	100 (100)				
500 (546) mg/L	45 (45)	100 (100)	100 (100)	100 (100)	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 72. Analytical chemistry data for 96-hour toxicity test on *Pimephales promelas* with boron.

Nominal (and Measured) Test Concentrations		Boron (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	0.03	25.0	8.01	8.0	290	104	144	ND
	Day 1		24.7	7.73	6.3				
	Day 2		24.8	7.54	5.9				
	Day 3		24.8	7.65	5.7				
	Day 4	0.04	24.7	7.95	6.0	278	100	132	
		0.035							
38.9 (46.1) mg/L	Day 0	54.7	25.0	8.16	8.0	318			
	Day 1		24.6	8.12	6.7				
	Day 2		24.8	8.02	6.4				
	Day 3		24.9	8.05	6.0				
	Day 4	37.4	24.7	8.07	6.4	323			
		46.1							
64.8 (71.5) mg/L	Day 0	82	25.0	8.14	7.9	335			
	Day 1		24.7	8.17	7.7				
	Day 2		24.8	8.05	4.6				
	Day 3		24.8	8.11	6.3				
	Day 4	61	24.7	8.02	5.4	346			
		71.5							
108 (123) mg/L	Day 0	146	25.0	8.18	8.0	362			
	Day 1		24.8	8.18	7.0				
	Day 2		25.0	8.15	6.5				
	Day 3		24.9	8.13	5.9				
	Day 4	99.1	24.8	8.15	6.7	376			
		123							
180 (200) mg/L	Day 0	223	25.0	8.18	8.0	406			
	Day 1		25.1	8.17	6.2				
	Day 2		24.9	8.14	6.1				
	Day 3		24.9	8.11	5.9				
	Day 4	176	24.8	8.13	5.3	422	146	136	
		200							
300 (352) mg/L	Day 0	388	25.0	8.17	8.0	476			
	Day 1		24.7	8.17	7.0				
	Day 2		24.8	8.17	6.8				
	Day 3		24.8	8.15	5.8				
	Day 4	316							
		352							
500 (546) mg/L	Day 0	585	25.0	8.14	8.0	597	316	168	ND
	Day 1		24.7	8.15	7.0				
	Day 2		24.8	8.13	7.4				
	Day 3								
	Day 4	507							
		546							

^a Boron Analysis EPA 200.8
ND Not Detect; below detection limit

32-day Toxicity of Boron on *Pimephales promelas*

The 32-day test to determine the toxicity of boron on *P. promelas* was completed by GLEC. The fish were continuously exposed for 32 days to five concentrations of boron (nominal concentrations of 6.3, 12.5, 25, 50 and 100 mg B/L) and to a dilution water control using a continuous flow-through system (Benoit et al. 1982). The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were four replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with < 24 hour old fertilized embryos. The embryos were randomly assigned to incubation cups until each incubation cup contained 30 embryos. The incubation cups were randomly assigned to the 2.5 L glass test chambers (1 cup per chamber) and suspended in the test solutions from a rocker arm assembly. The rocker arm assembly moves the incubation cups in a reciprocal motion within each test chamber. Embryos were inspected on a daily basis and the number of live, hatched and dead embryos was recorded. On Day 8 of the test (four days after first hatch), the surviving fish were randomly thinned to achieve 20 fish in each test chamber. The remainder of the surviving fish was discarded. The number of surviving fish was recorded at test termination (32 days). In addition, the wet weights were recorded for each fish at test termination. Because of the size range of fish in each test chamber, all of the fish from each test chamber were weighed together to determine average dry weight.

Instantaneous water temperature measurements made on September 12 (Day 2: 23.5°C-24.5°C), October 1 (Day 21: 23.7°C-24.3°C), October 9 (Day 29: 25.9°C-26.5°C), October 11 (Day 31: 23.7°C-24.1°C), and October 12 (Day 32: 23.8°C-24.5°C) exceeded the allowable range of 25 ± 1 °C in the toxicity testing method. However, the overall average water temperatures (across the duration of the test) in each replicate were within ± 0.5 °C of the target test temperature (25 °C) in all treatments. Therefore, the water temperature exceedances noted above likely had no effect on the results of this study.

On September 19 and 20, 2009 (test days 9 and 10) 60-90 percent mortality occurred in replicates one and two of the laboratory control. It is of GLEC's opinion that the equipment used during the thinning procedure on test day 8 contributed toxicity to these two control replicates, resulting in the high fish mortality. This high mortality was communicated to the EPA Work Assignment Manager and GLEC was advised to continue the test with the assumption that further control mortality would result in test failure. No further toxicity was observed in the remaining control fish throughout the test. However, because of the mortality observed in the control treatments, only replicates three and four of the laboratory control were used in the survival and growth statistical comparisons.

Once the test was complete, the LC₅₀, NOEC and LOEC values were determined using the average measured concentrations with the Spearman Karber and ANOVA methods. LC₂₅, LC₂₀ and LC₁₀ values were determined using the Probit method and EC₅₀, EC₂₅, EC₂₀ and EC₁₀ values were estimated using EPA's TRAP.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 73; test results are provided in Table 74. Survival and growth data are provided in Table 75 and analytical chemistry data are provided in Table 76. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 22.

Table 73. Test conditions for 32-day toxicity test on *Pimephales promelas* with boron.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (<24 hour fertilized embryos), GLEC Culture
2. Test Type and Duration:	Continuous flow-through; 32 days
3. Test Dates:	September 10-October 12, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	Live Brine Shrimp (<i>Artemia nauplii</i>) Twice daily
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume of Test Solutions:	2000 mL
10. No. of Test Organisms per Test Vessel:	30 eggs, thinned to 20 larvae after hatch
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	120 eggs, thinned to 80 larvae after hatch
13. Target or Nominal Test Concentrations (mg/L):	100, 50.0, 25.0, 12.5 and 6.25 mg/L-boron
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg/L):	112, 56.5, 27.4, 12.9, and 5.90 mg/L-nitrate
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-Chlorinated Lake Michigan Water
17. Test Material:	Boric Acid: Sigma Aldrich, ACS Reagent ≥ 99.5% Cas. No. 10043-35-3, Batch 118K0007 and Borax (sodium tetraborate decahydrate) Sigma Aldrich, ≥ 99.5%, ACS reagent, Cas. No. 1303-96-4, Lot # 118K0172
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Survival (LC ₅₀ , LC ₂₅ , LC ₂₀ and LC ₁₀ , NOEC and LOEC) and Growth (EC ₅₀ , EC ₂₅ , EC ₂₀ and EC ₁₀ , NOEC and LOEC)

Table 74. Test results for 32-day toxicity test on *Pimepehales promelas* with boron.

Results of a <u><i>Pimepehales promelas</i></u>		<u>32-Day Continuous Flow Chronic Toxicity Test</u>				
Conducted <u>09/10/09</u> - <u>10/12/09</u>		<u>Using: Boron (Boric Acid: Sigma Aldrich Cas. No. 10043-35-3</u> <u>Borax: Sigma Aldrich Cas. No. 1303-96-4)</u>				
Test Solution Concentrations Measured	Primary Control/ Dilution Water	5.90 mg/L	12.9 mg/L	27.4 mg/L	56.5 mg/L	112 mg/L
Embryo Percent Hatch (%)	100	100	100	100	100	100
32-Day Mean Survival (%)	97.5	93.8	92.5	58.8 ^a	1.3 ^a	0 ^a
32-Day Average Biomass ¹ (mg)	10.1	7.27 ^a	7.37 ^a	4.29 ^a	0.06 ^a	0 ^a
Embryo Percent Hatch NOEC:	100 mg/L		32-Day LC ₂₀ :	18.6 mg/L (15.9 mg/L – 21.3 mg/L)		
32-Day LC ₅₀ *:	28.4 mg/L (25.5 mg/L-31.7 mg/L)		32-Day LC ₁₀ :	12.8 mg/L (9.6 mg/L – 16.0 mg/L)		
32-Day Survival NOEC:	12.9 mg/L		32-Day EC ₅₀ :	28.7 mg/L (23.7 mg/L – 34.6 mg/L)		
32-Day Survival LOEC:	27.4 mg/L		32-Day EC ₂₅ :	25.1 mg/L (17.1 mg/L – 36.7 mg/L)		
32-Day Growth NOEC:	<5.9 mg/L		32-Day EC ₂₀ :	24.2 mg/L (14.3 mg/L – 41.0 mg/L)		
32-Day Growth LOEC:	5.9 mg/L		32-Day EC ₁₀ :	21.9 mg/L (8.6 mg/L – 56.0 mg/L)		
32-Day LC ₂₅ :	20.8 mg/L (18.2 mg/L – 23.4 mg/L)					

*: All LC, EC, NOEC and LOEC values are determined based on the average measured boron concentration.

NOEC: No-Observed-Effect-Concentration

LOEC: Lowest-Observed-Effect-Concentration

¹Biomass: Biomass is the average dry weight of the four replicates calculated by the total dry weight of surviving organisms divided by the initial number of organisms (20).

Table 75. Survival and growth data for 32-day toxicity test on *Pimephales promelas* with boron.

Nominal (and Measured) Test Concentration		Number of Eggs at Test Initiation	Number of Hatched Larvae	Number of Dead Eggs	Percent Hatched Larvae	Number of Larvae at Test Termination	Percent Survival at Test Termination*	Biomass ¹ (mg)
Dilution water/Control	Replicate # 1	30	30	0	100.0	1	5.0	1.27
	Replicate # 2	30	30	0	100.0	4	20.0	4.83
	Replicate # 3	30	30	0	100.0	19	95.0	9.62
	Replicate # 4	30	30	0	100.0	20	100.0	10.52
	Average				100.0		97.5 ²	10.07 ²
6.25 (5.9) mg/L	Replicate # 1	30	30	0	100.0	17	85.0	6.69
	Replicate # 2	30	30	0	100.0	18	90.0	6.84
	Replicate # 3	30	30	0	100.0	20	100.0	7.34
	Replicate # 4	30	30	0	100.0	20	100.0	8.21
	Average				100.0		93.8	7.27
12.5 (12.9) mg/L	Replicate # 1	30	30	0	100.0	17	85.0	6.99
	Replicate # 2	30	30	0	100.0	19	95.0	8.53
	Replicate # 3	30	30	0	100.0	19	95.0	6.77
	Replicate # 4	30	30	0	100.0	19	95.0	7.17
	Average				100.0		92.5	7.37
25.0 (27.4) mg/L	Replicate # 1	30	30	0	100.0	10	50.0	3.76
	Replicate # 2	30	30	0	100.0	10	50.0	3.62
	Replicate # 3	30	30	0	100.0	13	65.0	5.05
	Replicate # 4	30	30	0	100.0	14	70.0	4.73
	Average				100.0		58.8	4.29
50.0 (56.5) mg/L	Replicate # 1	30	30	0	100.0	0	0.0	0.00
	Replicate # 2	30	30	0	100.0	0	0.0	0.00
	Replicate # 3	30	30	0	100.0	1	5.0	0.25
	Replicate # 4	30	30	0	100.0	0	0.0	0.00
	Average				100.0		1.3	0.06
100 (112) mg/L	Replicate # 1	30	30	0	100.0	0	0.0	0.00
	Replicate # 2	30	30	0	100.0	0	0.0	0.00
	Replicate # 3	30	30	0	100.0	0	0.0	0.00
	Replicate # 4	30	30	0	100.0	0	0.0	0.00
	Average				100.0		0.0	0.00

* On Day 8 of the test, (four days after first hatch) the surviving fish were randomly thinned to 20 fish in each test chamber. Percent survival at test termination is the number of surviving at test termination divided by 20.

¹ Biomass: Biomass is the total dry weight of surviving organisms divided by the initial number of organisms (20)

² Due to a technician error on day 8, only replicates 3 and 4 were used in the growth and survival analysis for the laboratory control

Table 76. Analytical chemistry data for 32-day toxicity test on *Pimephales promelas* with boron.

Nominal (and Measured) Test Concentrations		Boron (mg/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmhos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate #	0.0	25.1		7.5				
	1	(0.0-0.1)	(23.7-26.0)	8.08	(7.0-8.1)	290	112	100	ND
	Replicate #	0.0	24.9		7.3				
	2	(0.0-0.1)	(23.8-26.3)	8.11	(6.8-7.8)	296	132	99	ND
	Replicate #	0.1	24.9		7.2				
	3	(0.0-0.4)	(24.2-26.0)	8.16	(6.9-7.4)	300	NM	NM	ND
	Replicate #	0.0	24.9		7.3				
	4	(0.0-0.1)	(24.1-25.5)	8.17	(7.1-7.5)	304	NM	NM	ND
Average Boron mg/L		0							
6.25 mg/L (5.9 mg/L)	Replicate #	5.8	24.6		7.5				
	1	(4.9-6.6)	(23.9-25.6)	8.16	(7.0-7.9)	301	128	106	ND
	Replicate #	6.0	24.9		7.3 (6.7)				
	2	(4.9-7.0)	(24.0-26.1)	8.17	8.0)	305	132	104	ND
	Replicate #	6.0	25.0		7.1				
	3	(5.0-7.1)	(24.1-26.2)	8.21	(6.6-7.5)	305	NM	NM	ND
	Replicate #	5.9	24.8		7.1				
	4	(5.1-6.6)	(23.9-25.4)	8.18	(6.7-7.6)	309	NM	NM	ND
Average Boron mg/L		5.9							
12.5 mg/L (12.9 mg/L)	Replicate #	13.0	24.8		7.4				
	1	(11.6-13.9)	(23.7-25.9)	8.17	(6.8-8.1)	307	132	110	ND
	Replicate #	13.1	24.9		7.5				
	2	(11.5-14.2)	(24.1-26.1)	8.20	(6.9-8.0)	308	130	107	ND
	Replicate #	13.1	25.1		7.3				
	3	(11.6-14.7)	(24.2-26.5)	8.25	(7.0-7.6)	310	NM	NM	ND
	Replicate #	12.3	25.0		7.4				
	4	(11.0-13.8)	(24.2-25.6)	8.21	(7.1-7.8)	313	NM	NM	ND
Average Boron mg/L		12.9							
25.0 mg/L (27.4 mg/L)	Replicate #	28.6	24.6		7.5				
	1	(23.8-34.3)	(23.7-25.7)	8.18	(7.1-8.0)	314	128	116	ND
	Replicate #	28.0	24.7		7.5				
	2	(25.1-32.5)	(23.8-26.2)	8.21	(7.2-8.0)	316	128	113	ND
	Replicate #	26.2	24.8		7.4				
	3	(22.7-31.1)	(23.8-26.1)	8.12	(7.1-7.8)	317	NM	NM	ND
	Replicate #	26.3	24.5		7.4				
	4	(22.9-31.2)	(23.5-25.2)	8.22	(7.1-7.7)	320	NM	NM	ND
Average Boron mg/L		27.4							
50.0 mg/L (56.5 mg/L)	Replicate #	56.1	24.8		7.6				
	1	(48.2-65.4)	(23.9-25.7)	8.21	(7.1-8.5)	331	132	128	ND
	Replicate #	56.4	24.7		7.5				
	2	(46.9-63.3)	(24.1-26.0)	8.23	(7.2-8.1)	333	130	127	ND
	Replicate #	57.4	24.8		7.5				
	3	(45.8-67.9)	(23.9-26.0)	8.27	(7.1-8.3)	351	NM	NM	ND
	Replicate #	56.2	24.8		7.5				
	4	(45.3-63.4)	(23.9-25.4)	8.24	(7.3-8.0)	337	NM	NM	ND
Average Boron mg/L		56.5							
100 mg/L (112 mg/L)	Replicate #	111	24.8		7.6				
	1	(98.9-117)	(23.6-26.0)	8.22	(7.0-8.4)	363	128	152	ND
	Replicate #	111	25.0		7.6				
	2	(93.7-117)	(23.9-26.2)	8.23	(7.2-8.4)	367	130	150	ND
	Replicate #	111	25.0		7.4				
	3	(97.2-122)	(24.4-26.2)	8.27	(7.0-8.0)	367	NM	NM	ND
	Replicate #	113	24.9		7.6				
	4	(103-123)	(24.0-25.6)	8.24	(7.3-8.0)	370	NM	NM	ND
Average Boron mg/L		112							

DO: Dissolved Oxygen
SC: Specific Conductance

ND: Non Detect; below detection limit.
NM: Not Measured

48-hr Toxicity of Boron (pH 7.75) on *Ceriodaphnia dubia*

The 48-hr test to determine the toxicity of boron (pH 7.75) on *C. dubia* was completed by GLEC. The *C. dubia* were continuously exposed for 48-hours to five concentrations of boron and to a dilution water control with a target test pH of 7.75 (range of 7.65 to 7.85) using a continuous flow-through system (modified Benoit mini-dilutor) and an in-line pH adjustment/metering unit. The pH of both the dilution water and stock solution were adjusted by using a 1:1 ratio of sulfuric acid and hydrochloric acid (acid solution). The pH adjusted stock solution was delivered to mixing cells and diluted with pH adjusted, de-chlorinated Lake Michigan water to achieve target nominal concentrations of boron and a target test pH of 7.75.

Due to the buffering capacity of the borax and boric acid solution used to prepare the boron concentrations, EPA agreed that GLEC should target the dilution water control pH at 7.75 (range of 7.65 to 7.85). The five test concentrations were targeted to a pH of ± 0.1 pH unit from the pH value defined at test initiation in each test concentration (i.e. regardless of whether or not the pH in the test concentrations were ± 0.1 pH units from that observed in the control water). The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were four replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with < 24 hour old *C. dubia* collected from the GLEC laboratory culture. Although these organisms were cultured in, and acclimated to, the dilution water's "natural" pH (typically between a pH of 7.9 and 8.2), they were not acclimated to the pH-adjusted dilution water prior to test initiation for two reasons: 1) GLEC does not maintain a laboratory culture of *C. dubia* in de-chlorinated Lake Michigan water maintained at a pH of 7.75 and 2) adequate acclimation of the organisms to the pH-adjusted dilution water would have jeopardized the age requirement (< 24 hour old at test initiation) for test organisms required under the toxicity testing method (ASTM 2007).

The *C. dubia* were randomly assigned to test cups until each test cup contained five *C. dubia*. The test cups were randomly assigned to the 2.5 L glass test chambers (1 cup per chamber) and suspended in the test solutions from a rod. *C. dubia* were counted on a daily basis and the number of live *C. dubia* was recorded. pH was recorded twice a day, at a minimum of eight hours apart (i.e. morning and evening). The number of surviving *C. dubia* was recorded at test termination (48-hours).

The test was completed at the following nominal boron concentrations: 25.0, 50.0, 100, 200, and 400 mg/L. The average pH for the dilution water control measured in the *C. dubia* toxicity test for the 48-hour test period was 7.77. The average pH over the 48-hour test duration for the five test concentrations of 27.6, 49.8, 118, 223, and 391 mg/L was 7.92, 8.03, 8.03, 8.07, and 8.06, respectively.

Once the test was complete, the LC₅₀ was determined using the average measured test concentrations with the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 77; test results are provided in Table 78. Analytical chemistry data are provided in Table 79. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 23.

Table 77. Test conditions for 48-hour toxicity test on *Ceriodaphnia dubia* with boron (pH 7.75).

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Ceriodaphnia dubia</i> , < 24 hours old, GLEC Culture
2. Test Type and Duration:	Continuous flow-through, 48 hours
3. Test Dates:	October 23-October 25, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume Test Solutions:	2000 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg/L):	400, 200, 100, 50.0, and 25.0
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg/L):	391, 223, 118, 49.8, and 27.6
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-Chlorinated Lake Michigan Water
17. Test Material:	Boric Acid: Sigma Aldrich, ACS Reagent ≥ 99.5% Cas. No. 10043-35-3, Batch 118K0007 and Borax (sodium tetraborate decahydrate) Sigma Aldrich, ≥ 99.5%, ACS reagent, Cas. No. 1303-96-4, Lot # 118K0172
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 78. Test results for 48-hour toxicity test on *Ceriodaphnia dubia* with boron at pH 7.75.

Results of a <u><i>Ceriodaphnia dubia</i></u> <u>48-Hour Static Acute Toxicity Test</u> Conducted <u>10/23/09</u> - <u>10/25/09</u> Using: <u>Boron (Boric Acid: Sigma Aldrich Cas No. 10043-35-3)</u> (Borax: <u>Sigma Aldrich Cas No. 1303-96-4</u>)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)			172	76.9	NA	NA
25.0 (27.6) mg/L	0 (0)	0 (0)			48-Hour LC ₅₀ * = 76.9 mg/L			
50.0 (49.8) mg/L	0 (0)	25 (25)			LC ₅₀ * 95% Confidence Limits			
100 (118) mg/L	15 (15)	75 (75)			24-Hr	48-Hr	72-Hr	96-Hr
200 (223) mg/L	75 (75)	100 (100)			LL 144	62.3	NA	NA
400 (391) mg/L	100 (100)	100 (100)			UL 202	94.9	NA	NA
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 79. Analytical chemistry data for 48-hour toxicity test on *Ceriodaphnia dubia* with boron at pH 7.75.

Nominal (and Measured) Test Concentrations		Boron (mg/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmhos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate # 1	0.05 (0.04-0.05)	24.6 (24.5-24.6)	7.65 (7.53-7.75)	7.7 (6.5-8.7)	320	146	47	ND
	Replicate # 2	0.06 (0.04-0.07)	25.0 (24.9-25.1)	7.75 (7.70-7.82)	7.9 (6.9-8.6)	328			
	Replicate # 3	0.05 (0.03-0.07)	25.2 (25.1-25.2)	7.84 (7.77-7.94)	7.7 (7.1-8.1)	322			
	Replicate # 4	0.06 (0.05-0.07)	24.9 (24.9-24.9)	7.71 (7.56-7.79)	7.5 (6.4-8.1)				
	Average	0.05	24.8	7.77	7.7	323	146	47	ND
25 mg/L (27.6 mg/L)	Replicate # 1	27.9 (27.2-28.5)	24.7 (24.5-24.8)	7.89 (7.80-7.95)	7.3 (5.9-8.1)	346	138	50	ND
	Replicate # 2	27.3 (25.9-28.0)	25.1 (25.0-25.2)	7.96 (7.89-8.06)	7.7 (7.2-8.0)	350			
	Replicate # 3	27.4 (27.1-27.8)	24.8 (24.7-24.9)	7.92 (7.87-8.00)	7.6 (6.7-8.1)				
	Replicate # 4	28.0 (27.2-28.9)	24.8 (24.8-24.9)	7.97 (7.89-8.06)	7.7 (7.1-8.1)				
	Average	27.6	24.7	7.92	7.6	348	138	50	ND
50 mg/L (49.8 mg/L)	Replicate # 1	49.8 (47.4-52.9)	24.6 (24.5-24.7)	8.00 (7.96-8.07)	7.4 (6.4-7.9)	364	144	57	ND
	Replicate # 2	50.1 (47.8-53.7)	24.8 (24.7-24.8)	8.04 (7.97-8.11)	7.7 (7.1-8.0)	365			
	Replicate # 3	49.3 (47.5-52.7)	24.8 (24.7-24.9)	8.00 (7.96-8.07)	7.8 (7.3-8.1)				
	Replicate # 4	49.8 (47.8-53.5)	24.8 (24.7-24.9)	8.04 (7.97-8.11)	7.5 (6.5-8.1)				
	Average	49.8	24.6	8.03	7.6	364	144	57	ND
100 mg/L (118 mg/L)	Replicate # 1	120 (111-127)	24.8 (24.6-24.9)	8.07 (8.03-8.13)	7.5 (6.7-8.0)	411	128	77	ND
	Replicate # 2	120 (112-124)	24.9 (24.8-25.0)	8.08 (8.02-8.14)	7.6 (6.8-8.0)	412			
	Replicate # 3	118 (110-130)	24.8 (24.7-24.9)	8.07 (8.02-8.13)	7.5 (6.4-8.1)				
	Replicate # 4	115 (105-125)	24.9 (24.6-25.1)	8.07 (8.00-8.14)	7.6 (6.9-8.0)				
	Average	118	24.8	8.03	7.5	411	128	77	ND
200 mg/L (223 mg/L)	Replicate # 1	227 (221-232)	24.7 (24.6-24.8)	8.08 (8.02-8.15)	7.7 (7.2-8.0)	475	132	105	ND
	Replicate # 2	222 (216-228)	24.5 (24.4-24.7)	8.08 (8.03-8.15)	7.5 (6.6-8.0)	477			
	Replicate # 3	218 (211-224)	24.7 (24.6-24.8)	8.09 (8.02-8.15)	7.7 (7.1-8.0)				
	Replicate # 4	225 (222-228)	25.1 (24.8-25.3)	8.07 (8.01-8.14)	7.6 (6.7-8.1)				
	Average	223	24.8	8.07	7.6	476	132	105	ND
400 mg/L (391 mg/L)	Replicate # 1	392 (381-399)	24.5 (24.4-24.6)	8.06 (8.00-8.13)	7.6 (6.7-8.0)	587	132	167	ND
	Replicate # 2	391 (386-396)	24.6 (24.4-24.8)	8.07 (8.01-8.14)	7.7 (7.1-8.0)	589			
	Replicate # 3	391 (388-394)	24.5 (24.3-24.8)	8.07 (8.01-8.14)	7.7 (7.2-8.0)				
	Replicate # 4	391 (384-401)	24.6 (24.4-24.7)	8.06 (8.01-8.13)	7.5 (6.4-8.0)				
	Average	391	24.6	8.06	7.6	588	132	167	ND

DO: Dissolved Oxygen
SC: Specific Conductance

ND: Non Detect; below detection limit.
NM: Not Measured

96-hour Toxicity of Boron (pH 6.75) on *Pimephales promelas*

The 96-hr test to determine the toxicity of boron (pH 6.75) on *P. promelas* was completed by GLEC. The *P. promelas* (collected from the GLEC laboratory culture) were continuously exposed for 96-hours to five concentrations of boron and to a dilution water control with a target test pH of 6.75 (range of 6.65 to 6.85) using a continuous flow-through system (modified Benoit mini-dilutor) and an in-line pH adjustment/metering unit. The pH of both the dilution water and stock solution were adjusted by using a 1:1 ratio sulfuric acid and hydrochloric acid (acid solution). The pH adjusted stock solution was delivered to mixing cells and diluted with pH adjusted Lake Michigan water to achieve target nominal concentrations of boron and a target test pH of 6.75. Due to the buffering capacity of the borax and boric acid solution used to prepare the boron concentrations, EPA agreed that GLEC should target to a pH of ± 0.1 pH unit from the pH value defined at test initiation in each test concentration (i.e. regardless of whether or not the pH in the test concentrations were ± 0.1 pH unit from that observed in the control water). The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were two replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with the *P. promelas*. Although these organisms were cultured in, and acclimated to, the dilution water's "natural" pH (typically between a pH of 7.9 and 8.2), they were not acclimated to the pH-adjusted dilution water prior to test initiation for two reasons: 1) GLEC does not maintain a laboratory culture of *P. promelas* in de-chlorinated Lake Michigan water maintained at a pH of 6.75 and 2) adequate acclimation of the organisms to the pH-adjusted dilution water may have jeopardized the weight requirement (0.1–5 g at test initiation) for test organisms required under the toxicity testing method (ASTM 2007).

The *P. promelas* were randomly assigned to the 2.5 L glass test chambers until each test chamber contained ten *P. promelas*. *P. promelas* were counted on a daily basis and the number of live *P. promelas* was recorded. pH was recorded twice a day, at a minimum of eight hours apart (i.e., morning and evening). The number of surviving *P. promelas* was recorded at test termination (96-hours).

The test was completed at the following nominal boron concentrations: 25.0, 50.0, 100, 200, and 400 mg/L (dilution factor of 0.5). The average pH for the dilution water control measured in the *P. promelas* toxicity test for the 96-hour test period was 6.67. The average pH over the 96-hour test duration for the five test concentrations of 32.9, 55.2, 122, 224, and 394 mg/L was 6.85, 6.93, 7.18, 7.28, and 7.33, respectively.

The hourly water temperatures as recorded by the continuous temperature logger did show that test temperatures fell outside the temperature allowance in the early morning on October 29, 2009 (readings of 23.8 and 23.9 °C). However, instantaneous water

temperatures measured by GLEC technicians on October 28 and later in the morning on October 29 never fell outside the acceptable range as outlined in the method. Therefore, these water temperature exceedances were very brief and likely had no effect on the results of this study.

Once the test was complete, the LC₅₀ was determined using the average measured test concentrations with the Probit and Spearman method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 80; test results are provided in Table 81. Analytical chemistry data are provided in Table 82. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 24.

Table 80. Test conditions for 96-hour toxicity test on *Pimephales promelas* with

boron (pH 6.75).

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (weight 0.11g and 18.4 mm length), GLEC Culture
2. Test Type and Duration:	Continuous flow-through, 96 hours
3. Test Dates:	October 29-November 02, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume of Test Solutions:	2000 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg/L):	400, 200, 100, 50.0, and 25.0
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg/L):	394, 224, 122, 55.2, and 32.9
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Boric Acid: Sigma Aldrich, ACS Reagent ≥ 99.5% Cas. No. 10043-35-3, Batch 118K0007 and Borax (sodium tetraborate decahydrate) Sigma Aldrich, ≥ 99.5%, ACS reagent, Cas. No. 1303-96-4, Lot # 118K0172
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 81. Test results for 96-hour toxicity test on *Pimephales promelas* with boron at pH 6.75.

Results of a <i>Pimephales promelas</i> 96-Hour Static Acute Toxicity Test Conducted <u>10/29/09</u> - <u>11/02/09</u> Using: Boron (Boric Acid: Sigma Aldrich Cas No. 10043-35-3) (Borax: Sigma Aldrich Cas No. 1303-96-4)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ * Values (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	>394	297	163	70.6
25.0 (32.9) mg/L	5 (5)	5 (5)	10 (10)	10 (10)	96-Hour LC ₅₀ * = 70.6 mg/L			
50.0 (55.2) mg/L	5 (5)	5 (5)	5 (5)	20 (20)				
					LC ₅₀ *95% Confidence Limits			
100 (122) mg/L	0 (0)	0 (0)	30 (30)	90 (90)	24-Hr	48-Hr	72-Hr	96-Hr
200 (224) mg/L	5 (5)	5 (5)	65 (65)	100 (100)	LL NA	285	130	58.3
					UL NA	310	205	86.3
400 (394) mg/L	10 (10)	95 (95)	100 (100)	100 (100)				
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit and Spearman			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 82. Analytical chemistry data for 96-hour toxicity test on *Pimephales promelas* with boron at pH 6.75.

Nominal (and Measured) Test Concentrations		Boron (mg/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate #	0.04	24.4	6.62	7.3				
	1	(0.04-0.04)	(24.0-24.8)	(6.48-6.69)	(6.7-8.0)	327	130	70	ND
	Replicate #	0.06	24.5	6.73	7.2				
	4	(0.05-0.06)	(24.0-24.8)	(6.63-6.88)	(6.7-8.3)	343			
Average		0.05	24.5	6.67	7.3	332	130	7	ND
25 mg/L (32.9 mg/L)	Replicate #	32.8	24.5	6.82	7.2				
	1	(30.3-38.6)	(24.1-24.9)	(6.73-6.93)	(6.6-8.0)	362	130	10	ND
	Replicate #	33.1	24.6	6.88	7.6 (7.2)				
	3	(30.4-39.0)	(24.1-25.0)	(6.80-6.98)	8.2)	365			
Average		32.9	24.5	6.85	7.4	363	130	10	ND
50 mg/L (55.2 mg/L)	Replicate #	55.2	24.4	6.94	7.4				
	1	(51.8-62.5)	(24.0-24.6)	(6.86-7.04)	(6.8-8.0)	382	126	11	ND
	Replicate #	55.2	24.6	6.93	7.3				
	3	(51.1-61.1)	(24.3-24.8)	(6.83-7.04)	(6.5-8.1)	351			
Average		55.2	24.5	6.93	7.4	371	126	11	ND
100 mg/L (122 mg/L)	Replicate #	120	24.5	7.17	7.4				
	1	(112-126)	(24.0-25.1)	(7.11-7.25)	(6.8-8.1)	440	130	18	ND
	Replicate #	123	24.5	7.19	7.6				
	3	(119-129)	(24.1-24.9)	(7.13-7.26)	(7.1-8.3)	447			
Average		122	24.5	7.18	7.5	442	130	18	ND
200 mg/L (224 mg/L)	Replicate #	223	24.3	7.28	7.2				
	2	(219-234)	(24.0-24.9)	(7.22-7.35)	(6.5-8.2)	518	128	25	ND
	Replicate #	224	25.3	7.28	7.3				
	4	(217-232)	(25.1-25.5)	(7.20-7.35)	(6.8-8.2)	527			
Average		224	24.8	7.28	7.2	521	128	25	ND
400 mg/L (394 mg/L)	Replicate #	391	24.3	7.33	7.4				
	1	(372-407)	(24.0-24.6)	(7.26-7.38)	(6.9-8.1)	670	126	41	ND
	Replicate #	397	24.6	7.32	7.5				
	4	(368-426)	(24.3-25.1)	(7.24-7.37)	(7.1-8.2)	696			
Average		394	24.4	7.33	7.4	678	126	41	ND

DO: Dissolved Oxygen
SC: Specific Conductance

ND: Non Detect; below detection limit.
NM: Not Measured

96-hr Toxicity of Boron (pH 7.75) on *Pimephales promelas*

The 96-hr test to determine the toxicity of boron (pH 7.75) on *P. promelas* was completed by GLEC. The *P. promelas* (collected from the GLEC laboratory culture) were continuously exposed for 96-hours to five concentrations of boron and to a dilution water control with a target test pH of 7.75 (range of 7.65 to 7.85) using a continuous flow-through system (modified Benoit mini-dilutor) and an in-line pH adjustment/metering unit. The pH of both the dilution water and stock solution were adjusted by using a 1:1 ratio of sulfuric acid and hydrochloric acid (acid solution). The pH adjusted stock solution was delivered to mixing cells and diluted with pH adjusted Lake Michigan water to achieve target nominal concentrations of boron and a target test pH of 7.75. Due to the buffering capacity of the borax and boric acid solution used to prepare the boron concentrations, EPA agreed that GLEC should target the dilution water control pH at 7.75 (range of 7.65 to 7.85). The five test concentrations were targeted to a pH of ± 0.1 pH unit from the pH value defined at test initiation in each test concentration (i.e. regardless of whether or not the pH in the test concentrations were ± 0.1 pH unit from that observed in the control water). The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were two replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with the *P. promelas*. Although these organisms were cultured in, and acclimated to, the dilution water's "natural" pH (typically between a pH of 7.9 and 8.2), they were not acclimated to the pH-adjusted dilution water prior to test initiation for two reasons: 1) GLEC does not maintain a laboratory culture of *P. promelas* in de-chlorinated Lake Michigan water maintained at a pH of 7.75 and 2) adequate acclimation of the organisms to the pH-adjusted dilution water may have jeopardized the weight requirement (0.1–5 g at test initiation) for test organisms required under the toxicity testing method (ASTM 2007).

The *P. promelas* were randomly assigned to the 2.5 L glass test chambers until each test chamber contained ten *P. promelas*. *P. promelas* were counted on a daily basis and the number of live *P. promelas* was recorded. pH was recorded twice a day, at a minimum of eight hours apart (i.e. morning and evening). The number of surviving *P. promelas* was recorded at test termination (96-hours).

The test was completed at the following nominal boron concentrations: 25.0, 50.0, 100, 200, and 400 mg/L (dilution factor of 0.5). The average pH for the dilution water control measured in the *P. promelas* toxicity test for the 96-hour test period was 7.68. The average pH over the 96-hour test duration for the five test concentrations of 28.6, 50.9, 121, 223, and 392 mg/L was 7.88, 7.98, 8.05, 8.07, and 8.06, respectively.

Once the test was complete, the LC₅₀ was determined using the average measured test concentrations with the Spearman method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 83; test results are provided in Table 84. Analytical chemistry data are provided in Table 85. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 25.

Table 83. Test conditions for 96-hour toxicity test on *Pimephales promelas* with boron (pH 7.75).

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (weight 0.12g and 22.0 mm length), GLEC Culture
2. Test Type and Duration:	Continuous flow-through, 96 hours
3. Test Dates:	October 23-October 27, 2009
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume of Test Solutions:	2000 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg/L):	400, 200, 100, 50.0, and 25.0
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg/L):	392, 223, 121, 50.9, and 28.6
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Boric Acid: Sigma Aldrich, ACS Reagent ≥ 99.5% Cas. No. 10043-35-3, Batch 118K0007 and Borax (sodium tetraborate decahydrate) Sigma Aldrich, ≥ 99.5%, ACS reagent, Cas. No. 1303-96-4, Lot # 118K0172
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 84. Test results for 96-hour toxicity test on *Pimephales promelas* with boron at pH 7.75.

Results of a <i>Pimephales promelas</i> 96-Hour Static Acute Toxicity Test								
Conducted <u>10/23/09</u> - <u>10/27/09</u>					Using: Boron (Boric Acid: Sigma Aldrich Cas No. 10043-35-3) (Borax: Sigma Aldrich Cas No. 1303-96-4)			
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ * Values (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	>392	289	202	137
25.0 (28.6) mg/L	0 (0)	0 (0)	0 (0)	0 (0)	96-Hour LC ₅₀ * = 137 mg/L			
50.0 (50.9) mg/L	0 (0)	0 (0)	0 (0)	0 (5)				
					LC ₅₀ *95% Confidence Limits			
100 (121) mg/L	0 (0)	0 (0)	0 (0)	21 (21)	24-Hr	48-Hr	72-Hr	96-Hr
200 (223) mg/L	5 (5)	10 (10)	65 (65)	100 (100)	LL NA	260	178	118
					UL NA	320	229	158
400 (392) mg/L	10 (10)	95 (95)	100 (100)	100 (100)	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Spearman			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 85. Analytical chemistry data for 96-hour toxicity test on *Pimephales promelas* with boron at pH 7.75.

Nominal (and Measured) Test Concentrations		Boron (mg/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmhos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate #	0.05	24.8	7.63	7.3				
	1	(0.04-0.05)	(24.5-25.3)	(7.49-7.75)	(6.5-8.0)	351	146	41	ND
	Replicate #	0.06	25.1	7.72	7.3				
	4	(0.05-0.07)	(24.9-25.4)	(7.56-7.85)	(6.4-8.1)	321			
Average		0.05	24.9	7.68	7.3	336	146	41	ND
25 mg/L (28.4 mg/L)	Replicate #	28.7	24.9	7.86	7.2				
	1	(27.2-31.2)	(24.5-25.4)	(7.70-7.95)	(5.9-8.1)	375	138	49	ND
	Replicate #	28.6	25.0	7.91	7.4				
	3	(27.1-32.1)	(24.7-25.4)	(7.80-8.00)	(6.7-8.1)	345			
Average		28.6	24.9	7.88	7.3	360	138	49	ND
50 mg/L (50.9 mg/L)	Replicate #	51.0	24.7	7.97	7.2				
	1	(47.4-54.7)	(24.5-25.1)	(7.80-8.07)	(6.4-8.0)	393	146	57	ND
	Replicate #	50.9	24.9	7.98	7.4				
	3	(47.5-55.5)	(24.7-25.4)	(7.90-8.07)	(6.7-8.1)	361			
Average		50.9	24.8	7.98	7.3	377	146	57	ND
100 mg/L (121 mg/L)	Replicate #	121	24.9	8.06	7.4				
	1	(111-127)	(24.6-25.3)	(7.97-8.13)	(6.7-8.1)	436	128	77	ND
	Replicate #	121	25.0	8.05	7.1				
	3	(110-130)	(24.7-25.4)	(7.96-8.13)	(6.2-8.1)	410			
Average		121	24.9	8.05	7.3	423	128	77	ND
200 mg/L (223 mg/L)	Replicate #	222	24.6	8.07	7.3				
	2	(216-228)	(24.4-25.1)	(7.99-8.15)	(6.6-8.0)	500	132	107	ND
	Replicate #	225	25.2	8.07	7.3				
	4	(222-228)	(24.8-25.6)	(8.00-8.14)	(6.7-8.1)	476			
Average		223	24.9	8.07	7.3	488	132	107	ND
400 mg/L (392 mg/L)	Replicate #	392	24.7	8.07	7.3				
	1	(381-399)	(24.4-25.1)	(8.00-8.13)	(6.7-8.0)	616	128	168	ND
	Replicate #	392	24.7	8.06	7.3				
	4	(384-401)	(24.4-25.2)	(8.01-8.13)	(6.4-8.0)	590			
Average		392	24.7	8.06	7.3	603	128	168	ND

DO: Dissolved Oxygen
SC: Specific Conductance

ND: Non Detect; below detection limit.
NM: Not Measured

96-hr Toxicity of Boron (pH 8.75) on *Pimephales promelas*

The 96-hr test to determine the toxicity of boron (pH 8.75) on *P. promelas* was completed by GLEC. The *P. promelas* (collected from the GLEC laboratory culture) were continuously exposed for 96-hours to five concentrations of boron and to a dilution water control with a target test pH of 8.75 (range of 8.65 to 8.85) using a continuous flow-through system (modified Benoit mini-dilutor) and an in-line pH adjustment/metering unit. The stock solutions used for the test were prepared by diluting a known weight (grams) of borax and boric acid to a known volume of dilution water (de-chlorinated Lake Michigan water). The pH of both the dilution water and stock solution were adjusted by using sodium hydroxide. The pH adjusted stock solution was delivered to mixing cells and diluted with pH adjusted Lake Michigan water to achieve target nominal concentrations of boron and a target test pH of 8.75. The temperature-controlled test concentration solutions were supplied to each test chamber via the continuous flow-through system at a rate of approximately four turnovers a day. There were two replicate test chambers for each treatment. The flow through test was conducted at 25 ± 1 °C with a photoperiod of 16 hr light and 8 hr darkness (ambient laboratory light).

After test concentrations had achieved steady state in the flow through system, the test was initiated with the *P. promelas*. Although these organisms were cultured in, and acclimated to, the dilution water's "natural" pH (typically between a pH of 7.9 and 8.2), they were not acclimated to the pH-adjusted dilution water prior to test initiation for two reasons: 1) GLEC does not maintain a laboratory culture of *P. promelas* in de-chlorinated Lake Michigan water maintained at a pH of 8.75 and 2) adequate acclimation of the organisms to the pH-adjusted dilution water may have jeopardized the weight requirement (0.1–5 g at test initiation) for test organisms required under the toxicity testing method (ASTM 2007).

The *P. promelas* were randomly assigned to the 2.5 L glass test chambers until each test chamber contained ten *P. promelas*. *P. promelas* were counted on a daily basis and the number of live *P. promelas* was recorded. pH was recorded twice a day, at a minimum of eight hours apart (i.e., morning and evening). The number of surviving *P. promelas* was recorded at test termination (96-hours).

The test was completed at the following nominal boron concentrations: 25.0, 50.0, 100, 200, and 400 mg/L (dilution factor of 0.5). The average pH for the dilution water control measured in the *P. promelas* toxicity test for the 96-hour test period was 8.75. The average pH over the 96-hour test duration for the five test concentrations of 21.1, 42.4, 112, 219, and 376 mg/L was 8.72, 8.70, 8.70, 8.70, and 8.67, respectively.

Instantaneous water temperature measurements made on February 13, 14, 15 and 16 (Days 1-4: 23.6°C-24.7°C) exceeded the allowable range of 25 ± 1 °C outlined in the toxicity testing method. However, the average water temperatures across the duration of the test in each replicate were always ± 0.9 °C of the target test temperature (25 °C) in all treatments. In addition, the water temperature as recorded by the continuous temperature logger did show that the test temperature fell outside the temperature allowance during

the times of 0430 to 0830 on those days. However, due to the relatively small number of temperature readings measured outside the range, these water temperature exceedances likely had little effect on the results of this study.

Once the test was complete, the LC₅₀ was determined using the average measured test concentrations with the Probit method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 86; test results are provided in Table 87. Analytical chemistry data are provided in Table 88. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 26.

Table 86. Test conditions for 96-hour toxicity test on *Pimephales promelas* with

boron (pH 8.75).

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Pimephales promelas</i> , (weight 0.13g and 22.8 mm length), GLEC Culture
2. Test Type and Duration:	Continuous flow-through, 96 hours
3. Test Dates:	February 12-February 16, 2010
4. Test Temperature (°C):	25 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m ² /s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	2.5 Liter glass Tank
9. Volume of Test Solutions:	2000 mL
10. No. of Test Organisms per Test Vessel:	10
11. No. of Test Vessels per Treatment:	2
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg/L):	400, 200, 100, 50.0, and 25.0
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg/L):	376, 219, 112, 42.4, and 21.1
15. Renewal of Test Solutions:	Continuous flow through, 4 turnovers per day
16. Dilution and Primary Control Water:	De-chlorinated Lake Michigan Water
17. Test Material:	Boric Acid: Sigma Aldrich, ACS Reagent ≥ 99.5% Cas. No. 10043-35-3, Batch 118K0007 and Borax (sodium tetraborate decahydrate) Sigma Aldrich, ≥ 99.5%, ACS reagent, Cas. No. 1303-96-4, Lot # 118K0172
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 87. Test results for 96-hour toxicity test on *Pimephales promelas* with boron at pH 8.75.

Results of a <i>Pimephales promelas</i> 96-Hour Static Acute Toxicity Test											
Conducted 02/12/10 - 02/16/10					Using: Boron (Boric Acid: Sigma Aldrich Cas No. 10043-35-3) (Borax: Sigma Aldrich Cas No. 1303-96-4)						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ * Values (mg/L)						
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr			
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	5 (5)	>373 325 195 133						
25.0 (21.1) mg/L	0 (0)	0 (0)	0 (0)	0 (0)							
50.0 (42.4) mg/L	0 (0)	0 (0)	0 (0)	0 (0)	96-Hour LC ₅₀ *= 133 mg/L						
100 (112) mg/L	0 (0)	0 (0)	5 (5)	30 (30)	LC ₅₀ *95% Confidence Limits						
200 (219) mg/L	5 (5)	15 (15)	60 (60)	95 (95)	24-Hr	48-Hr	72-Hr	96-Hr			
400 (376) mg/L	10 (10)	65 (65)	100 (100)	100 (100)	LL NA 276 165 110 UL NA 412 227 157						
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable						
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit						

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 88. Analytical chemistry data for 96-hour toxicity test on *Pimephales promelas* with boron at pH 8.75.

Nominal (and Measured) Test Concentrations		Boron (mg/L)	Temperature (°C)	pH (s.u.)	DO (mg/L)	SC (mmos)	Hardness (mg/L)	Alkalinity (mg/L)	Ammonia (mg/L)
Dilution Water/Control	Replicate #	0.04	24.2	8.74	7.6				
	1	(0.03-0.04)	(23.8-24.6)	(8.68-8.83)	(7.4-7.7)	290	126	111	ND
	Replicate #	0.03	24.4	8.75	7.4				
	2	(0.03-0.03)	(23.8-25.2)	(8.71-8.81)	(7.1-7.6)	299			
Average		0.03	24.3	8.75	7.5	295	126	111	ND
25 mg/L (21.1 mg/L)	Replicate #	21.9	24.3	8.72	7.5				
	1	(21.7-22.2)	(23.8-24.8)	(8.67-8.77)	(7.4-7.7)	331	110	133	ND
	Replicate #	20.2	24.2	8.71	7.3				
	3	(18.0-21.7)	(23.8-25.2)	(8.63-8.77)	(7.1-7.4)	336			
Average		21.1	24.2	8.72	7.4	333	110	133	ND
50 mg/L (42.4 mg/L)	Replicate #	42.6	24.3	8.69	7.1				
	2	(37.0-46.0)	(23.8-25.2)	(8.62-8.72)	(6.8-7.6)	368	116	156	ND
	Replicate #	42.7	24.3	8.72	7.2				
	4	(36.3-45.4)	(23.8-24.8)	(8.67-8.77)	(7.1-7.4)	370			
Average		42.4	24.3	8.70	7.2	369	116	156	ND
100 mg/L (112 mg/L)	Replicate #	111	24.3	8.71	7.3				
	1	(93.5-125)	(23.9-25.2)	(8.65-8.76)	(7.1-7.5)	472	116	235	ND
	Replicate #	113	24.3	8.70	7.4				
	4	(96.3-128)	(23.6-25.0)	(8.63-8.76)	(7.2-7.6)	464			
Average		112	24.3	8.70	7.3	471	116	235	ND
200 mg/L (219 mg/L)	Replicate #	207	24.3	8.70	7.3				
	1	(185-254)	(23.6-24.8)	(8.64-8.73)	(7.0-7.6)	628	120	340	ND
	Replicate #	231	24.5	8.70	7.2				
	4	(202-264)	(23.9-25.2)	(8.64-8.74)	(7.1-7.4)	638			
Average		219	24.4	8.70	7.3	633	120	340	ND
400 mg/L (376 mg/L)	Replicate #	382	24.1	8.67	7.2				
	1	(359-412)	(24.0-24.5)	(8.62-8.73)	(6.9-7.3)	844	108	510	ND
	Replicate #	370	24.1	8.67	7.2				
	4	(353-399)	(23.8-24.8)	(8.62-8.71)	(7.1-7.3)	845			
Average		376	24.1	8.67	7.2	845	108	510	ND

DO: Dissolved Oxygen
SC: Specific Conductance

ND: Non Detect; below detection limit.
NM: Not Measured

Manganese

Table 89 provides a summary of estimated LC₅₀ values for the two toxicity tests performed using manganese. LC₅₀ values ranged between 31.5 and 43.3 mg Mn/L.

Table 89. LC₅₀ estimates for toxicity tests performed using manganese.

Test Species and Duration	LC ₅₀ (mg Mn/L)
<i>Lampsilis siliquoidea</i> 96 hr	43.3
<i>Megaloniaias nervosa</i> - 96 hr	31.5

For each of the acute toxicity tests completed using manganese, two tables were generated: the first summarizes the test results for each toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity tests. Also discussed, if applicable, are deviations from the guidance provided in the ASTM method used to complete the toxicity testing.

96-hr Toxicity of Manganese on *Lampsilis silquoidea*

The 96-hr test to determine the toxicity of manganese on *L. silquoidea* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Missouri State University laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate. One replicate was mistakenly loaded with only 4 individuals, but this was accounted for in the LC₅₀ calculation.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 150, 75, 37, 18.8, 9.4, and 4.7 mg Mn/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 90; test results are provided in Table 91. Analytical chemistry data are provided in Table 92. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 27.

Table 90. Test conditions for 96-hour toxicity test on *Lampsilis siliquoidea* with manganese.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Lampsilis siliquoidea</i> , juveniles <5 days old, Missouri State University
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 8 - 12, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg Mn/L):	150, 75, 37, 18.8, 9.4, and 4.7
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- Mn mg/L):	154.9, 72.5, 34.5, 18.5, 10.1 and 4.5
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Manganese sulfate monohydrate: Fisher Scientific, ACS grade assay, 98.7%, Cas. No. 7785-87-7, Lot # 086316 and manganese chloride tetrahydrate, Fisher Scientific, certified ACS Assay 99.8% Cas. No. 7773-01-5, Lot # 081484
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 91. Test results for 96-hour toxicity test on *Lampsilis siliquoidea* with manganese.

Results of a <i>Lampsilis siliquoidea</i>		96-Hour Static Acute Toxicity Test						
Conducted <u>09/08/09 - 09/12/09</u>		<u>Using: Manganese sulfate Cas. No. 7785-87-7 &manganese chloride Cas. No. 7773-01-5</u>						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	62.8	50.0	48.2	43.3
4.7 (4.5) mg/L	0	0	0	0	96-Hour LC ₅₀ * = 43.3 mg/L			
9.4 (10.1) mg/L	0	0	0	0	LC ₅₀ 95% Confidence Limits			
18.8 (18.5) mg/L	0	0	0	0	24-Hr	48-Hr	72-Hr	96-Hr
37 (34.5) mg/L	0	0	5.2	21.0	LL 52.7	NR	45.0	38.1
					UL 74.9	NR	51.7	49.2
75 (72.5) mg/L	70	100	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
150 (154.9) mg/L	95	100	100	100				
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 92. Analytical chemistry data for 96-hour toxicity test on *Lampsilis siliquoidea* with manganese.

Nominal (Measured) Test Concentration		Manganese ^a	Temperature	pH	D.O.	Conductivity	Alkalinity	Hardness
		(mg/L)	(°C)	(s.u.)	(mg/L)	(mmos)	(mg/L)	(mg/L)
Dilution water/Control	Day 0	<0.01	20.4	7.9	7.08	316	62	92
	Day 1		20.3					
	Day 2		20.4					
	Day 3		19.6					
	Day 4	<0.01	19.1	7.9	7.50	322	62	90
		na						
4.7 (4.5) mg/L	Day 0	4.5	20.5	7.9	7.83	324	62	98
	Day 1		20.2					
	Day 2		20.3					
	Day 3		19.5					
	Day 4	4.6	19.1	7.9	7.69	328	62	100
		4.5						
9.4 (10.1) mg/L	Day 0	9.2	20.4	7.9	7.87	341	62	110
	Day 1		20.3					
	Day 2		20.4					
	Day 3		19.6					
	Day 4	11.0	19.0	7.9	7.65	345	62	110
		10.1						
18.8 (18.5) mg/L	Day 0	19.0	20.4	7.7	7.83	376	62	120
	Day 1		20.2					
	Day 2		20.3					
	Day 3		19.6					
	Day 4	18.0	19.0	7.9	7.72	380	62	124
		18.5						
37 (34.5) mg/L	Day 0	34.0	20.5	7.7	7.94	447	62	152
	Day 1		20.2					
	Day 2		20.3					
	Day 3		19.6					
	Day 4	35.0	19.0	7.9	7.76	450	62	152
		34.5						
75 (72.5) mg/L	Day 0	73.0	20.5	7.7	7.88	582	62	220
	Day 1		20.2					
	Day 2		20.3					
	Day 3		19.7					
	Day 4	72.0	19.1	7.9	7.70	590	62	224
		72.5						
150 (154.9) mg/L	Day 0	150.0	20.5	7.7	7.88	840	62	*
	Day 1		20.3					
	Day 2		20.4					
	Day 3		19.7					
	Day 4	160.0	19.2	7.9	7.80	850	62	*
		154.9						

^a Manganese Analysis Method 200.7

* interference in hardness measurement

na = not applicable

96-hr Toxicity of Manganese on *Megalonaias nervosa*

The 96-hr test to determine the toxicity of manganese on *M. nervosa* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Genoa National Fish Hatchery, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate. In one replicate of the control a test organism was inadvertently crushed, but this was accounted for in the LC₅₀ calculation.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 300, 150, 75, 37.5, and 18.8 mg Mn/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 93; test results are provided in Table 94. Analytical chemistry data are provided in Table 95. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 28.

Table 93. Test conditions for 96-hour toxicity test on *Megaloniaias nervosa* with manganese.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Megaloniaias nervosa</i> , juveniles <5 days old (Genoa National Fish Hatchery)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	October 23 - 27, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg Mn/L):	300, 150, 75, 37.5, and 18.8
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg Mn/L):	290, 140, 72, 34, and 18
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Manganese sulfate monohydrate: Fisher Scientific, ACS grade assay, 98.7%, Cas. No. 7785-87-7, Lot # 086316 and manganese chloride tetrahydrate, Fisher Scientific, certified ACS Assay 99.8% Cas. No. 7773-01-5, Lot # 081484
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 94. Test results for 96-hour toxicity test on *Megaloniaias nervosa* with manganese.

Results of a <u><i>Megaloniaias nervosa</i></u>					<u>96-Hour Static Acute Toxicity Test</u>			
Conducted <u>10/23/09 - 10/27/09</u>					<u>Using: Manganese sulfate Cas. No. 7785-87-7 & manganese chloride Cas. No. 7773-01-5</u>			
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	41.6	37.5	31.5	31.5
18.8 (18) mg/L	0	0	0	0	96-Hour LC ₅₀ [*] = 31.5 mg/L			
37.5 (34) mg/L	30	40	65	65	LC ₅₀ 95% Confidence Limits			
75 (72) mg/L	95	100	100	100	24-Hr	48-Hr	72-Hr	96-Hr
150 (140) mg/L	100	100	100	100	LL 35.6	32.2	27.2	27.2
300 (290) mg/L	100	100	100	100	UL 48.8	43.6	36.6	36.6
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 95. Analytical chemistry data for 96-hour toxicity test on *Megalonaias nervosa* with manganese.

Nominal (Measured) Test Concentration		Manganese ^a (mg/L)	Temp. (°C)	pH (s.u.)	D.O. (mg/L)	Cond. (mmos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	<0.01	20.8	8.0	8.34	303	60	90	<0.05
	Day 1		20.9	8.1	8.04	297			
	Day 2		20.9	8.1	8.13	303			
	Day 3		20.9	8.0	8.31	305			
	Day 4 total	0.1	20.8	8.0	8.00	315	60	92	<0.05
	Day 4 dissolved	0.7							
		0.1							
18.8 (18.0) mg/L	Day 0	18.0	20.8	7.9	8.33	375	60	112	<0.05
	Day 1		20.9	7.9	8.10	362			
	Day 2		20.9	7.7	8.19	371			
	Day 3		20.9	7.6	8.31	379			
	Day 4 total	18.0	20.8	7.8	7.92	383	60	112	<0.05
	Day 4 dissolved	16.0							
		18.0							
37.5 (34.0) mg/L	Day 0	33.0	20.8	7.9	8.26	445	60	*	<0.05
	Day 1		20.9	7.7	8.11	442			
	Day 2		20.9	7.6	8.25	458			
	Day 3		20.9	7.6	8.42	478			
	Day 4 total	35.0	20.8	7.7	7.94	495	60	*	<0.05
	Day 4 dissolved	34.0							
		34.0							
75 (72.0) mg/L	Day 0	70.0	20.8	7.9	8.29	582	60	*	<0.05
	Day 1		20.9	7.7	8.07	569			
	Day 2		20.8	7.6	8.26	567			
	Day 3		20.9	7.5	8.32	581			
	Day 4 total	74.0	20.9	7.7	7.91	589	62	*	<0.05
	Day 4 dissolved	68.0							
		72.0							
150 (140.0) mg/L	Day 0	140.0	20.8	7.8	8.33	841	60	*	<0.05
	Day 1		20.9	7.7	8.03	826			
	Day 2		20.8	7.6	8.22	817			
	Day 3		20.9	7.5	8.40	851			
	Day 4 total	140.0	20.9	7.6	7.88	848	62	*	<0.05
	Day 4 dissolved	150.0							
		140.0							
300 (290) mg/L	Day 0	290.0	20.8	7.7	8.27	1333	60	*	<0.05
	Day 1		20.9	7.7	7.98	1301			
	Day 2		20.8	7.6	8.20	1300			
	Day 3		20.9	7.5	8.22	1325			
	Day 4 total	290.0	20.9	7.6	7.94	1340	62	*	<0.05
	Day 4 dissolved	290.0							
		290.0							

^aManganese Analysis Method 200.7

*interference in hardness measurement

Temp. = temperature; Cond. = conductivity

Fluoride

Table 96 provides a summary of estimated LC₅₀ values for the two toxicity tests performed using fluoride. LC₅₀ values ranged between 13.4 and 62.0 mg F/L.

Table 96. LC₅₀ estimates for toxicity tests performed using fluoride.

Test Species and Duration	LC ₅₀ (mg F/L)
<i>Sphaerium simile</i> - 96 hr	62.0
<i>Hyaella azteca</i> - 96 hr	13.4

For each of the acute toxicity tests completed using fluoride, two tables were generated: the first summarizes the test results for each toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity tests.

96-hr Toxicity of Fluoride on *Sphaerium simile*

The 96-hr test to determine the toxicity of fluoride on *S. simile* was completed by INHS. Test organisms, juveniles released from field collected adults, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 800, 400, 200, 100, and 50 mg F/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 97; test results are provided in Table 98. Analytical chemistry data are provided in Table 99. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 29.

Table 97. Test conditions for 96-hour toxicity test on *Sphaerium simile* with fluoride.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Sphaerium simile</i> , juveniles (released from field-collected adults)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	July 13 - 17, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	120 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg F/L):	800, 400, 200, 100, and 50
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg F/L):	800, 390, 185, 88 and 44
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium fluoride: Acros Organics, 99+% for analysis ACS, Cas. No. 7681-49-5, Lot # A0243428
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 98. Test results for 96-hour toxicity test on *Sphaerium simile* with fluoride.

Results of a <i>Sphaerium simile</i>		96-Hour Static Acute Toxicity Test						
Conducted 07/13/09 - 07/17/09		Using: Sodium fluoride: Sigma Aldrich Cas. No. 7681-49-5						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	5	>800	>800	>800	62
50 (44) mg/L	0	0	0	25	96-Hour LC ₅₀ * = 62 mg/L			
100 (88) mg/L	0	0	0	75				
					LC ₅₀ 95% Confidence Limits			
200 (185) mg/L	0	0	35	100	24-Hr	48-Hr	72-Hr	96-Hr
400 (390) mg/L	0	5	50	100	LL NR	NR	NR	51
					UL NR	NR	NR	75
800 (800) mg/L	0	5	25	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

- All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 99. Analytical chemistry data for 96-hour toxicity test *Sphaerium simile* with fluoride.

Nominal (Measured) Test Concentration		Fluoride ^a (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	<0.5	22.9	8.0	7.76	307	62	96
	Day 1		22.9		7.97			
	Day 2		22.8		8.00			
	Day 3		22.7		7.63			
	Day 4	<0.5	22.9	8.0	8.05	310	62	96
		na						
50 (44) mg/L	Day 0	45	22.8	8.1	7.82	555	64	94
	Day 1		22.8		7.92			
	Day 2		22.8		7.83			
	Day 3		22.7		7.70			
	Day 4	44	22.9	8.0	8.04	558	68	80
		44						
100 (88) mg/L	Day 0	86	22.8	8.1	7.80	785	66	80
	Day 1		22.9		8.11			
	Day 2		22.8		7.95			
	Day 3		22.7		7.67			
	Day 4	91	22.9	8.1	7.53	784	68	62
		88						
200 (185) mg/L	Day 0	190	22.9	8.0	7.82	1264	78	76
	Day 1		22.8		8.05			
	Day 2		22.9		7.82			
	Day 3		22.8		6.63			
	Day 4	180	22.9	8.1	6.03	1268	82	50
		185						
400 (390) mg/L	Day 0	400	22.9	8.2	7.78	2210	120	64
	Day 1		22.9		7.99			
	Day 2		22.8		7.79			
	Day 3		22.8		4.88			
	Day 4	380	23.0	7.9	5.49	2210	120	30
		390						
800 (800) mg/L	Day 0	800	23.0	8.2	7.82	4050	160	14
	Day 1		22.9		8.05			
	Day 2		22.9		8.02			
	Day 3		22.8		6.51			
	Day 4	800	22.9	8.0	6.89	4090	160	2
		800						

^a Fluoride Analysis Method 300.0

na = not applicable

96-hr Toxicity of Fluoride on *Hyaella azteca*

The 96-hr test to determine the toxicity of fluoride on *H. azteca* was completed by GLEC. *H. azteca* were collected from GLEC's laboratory culture. These organisms are maintained in 10 gallon glass aquaria; plastic artificial turf and screen mesh serve as a substrate for the culture. The tanks are filled with de-chlorinated Lake Michigan water (City of Traverse City, Michigan water passed through an activated carbon filter). Cultures are fed 50 mL of 4 g/L Tetrafin slurry daily. When visible algae are not observed within the glass aquaria, algae (*Selenastrum* sp.) are used as a supplement to the Tetrafin slurry. Additionally, on occasion, dried Aspen (*Populus* sp.) leaves are prepared as a food supplement. The culture is maintained in a 16-hour light: 8-hour dark photoperiod at a temperature between 23 and 26 °C.

Test organisms were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 7.8, 12.9, 21.6, 36.0, 60.0, and 100 mg F/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Probit and Spearman Karber methods.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 100; test results are provided in Table 101. Analytical chemistry data are provided in Table 102. Accompanying information, including raw laboratory data, analytical chemistry data, reference toxicant data and statistical analyses, is provided in Appendix 30.

Table 100. Test conditions for 96-hour toxicity test on *Hyalella azteca* with fluoride.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Hyalella azteca</i> , 14 days old, GLEC culture
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 17- September 21, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	100 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Target or Nominal Test Concentrations (mg F/L):	100, 60, 36, 21.6, 12.9, and 7.8
14. Analytical Test Concentrations (average of samples collected at test initiation and termination-mg F/L):	89.4, 58.9, 32.7, 22.8, 14.3, and 8.5
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium Fluoride: Sigma Aldrich, 99+% ACS Reagent Cas. No. 7681-49-4, Batch # 06810JJ
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 101. Test results for 96-hour toxicity test on *Hyalella azteca* with fluoride.

Results of a <i>Hyalella azteca</i> 96-Hour Static Acute Toxicity Test Conducted 09/17/09 - 09/21/09 Using: Fluoride (Sodium Fluoride Sigma Aldrich Cas. No. 7681-49-4)								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0 (0)	0 (0)	0 (0)	0 (0)	25.1	16.0	13.8	13.4
7.8 (8.5) mg/L	0 (0)	0 (0)	0 (0)	0 (0)	96-Hour LC₅₀* = 13.4 mg/L			
12.9 (14.3) mg/L	0 (0)	45 (45)	70 (70)	70 (70)	LC₅₀ 95% Confidence Limits			
21.6 (22.8) mg/L	20 (20)	80 (80)	90 (90)	95 (95)	24-Hr	48-Hr	72-Hr	96-Hr
36 (32.7) mg/L	100 (100)	100 (100)	100 (100)	100 (100)	LL 23.3	13.9	12.0	11.8
60 (58.9) mg/L	100 (100)	100 (100)	100 (100)	100 (100)	UL 27.1	18.3	15.6	15.2
100 (89.4) mg/L	100 (100)	100 (100)	100 (100)	100 (100)	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ Confidence Limit Values: Probit and Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ values are determined based on measured concentrations.

Table 102. Analytical chemistry data for 96-hour toxicity test on *Hyalella azteca* with fluoride.

Nominal (and Measured) Test Concentrations		Fluoride ^a (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)	Ammonia (mg/L)
Dilution water/Control	Day 0	ND	22.0	7.84	8.0	300	54	80	ND
	Day 1		21.9	7.79	7.5				
	Day 2		22.1	7.79	7.1				
	Day 3		22.0	7.84	7.9				
	Day 4	ND	22.2	7.81	8.0	325	68	88	
		ND							
7.8 (8.5) mg/L	Day 0	8.4	22.0	7.84	8.0	368			
	Day 1		22.0	7.78	7.5				
	Day 2		22.1	7.83	7.3				
	Day 3		22.0	7.91	7.7				
	Day 4	8.6	22.2	7.87	8.0	363			
		8.5							
12.9 (14.3) mg/L	Day 0	14.1	22.0	7.85	8.0	393			
	Day 1		22.0	7.85	7.5				
	Day 2		22.1	7.88	7.4				
	Day 3		21.9	7.97	8.1				
	Day 4	14.5	22.2	7.89	8.0	377			
		14.3							
21.6 (22.8) mg/L	Day 0	22.0	22.0	7.85	8.0	432			
	Day 1		21.9	7.90	7.5				
	Day 2		22.0	7.89	7.3				
	Day 3		21.8	8.02	8.0				
	Day 4	23.5	21.9	7.97	8.0	422	66	100	
		22.8							
36 (32.7) mg/L	Day 0	33.4	22.0	7.88	8.0	502			
	Day 1	32.0	21.9	7.91	7.5				
	Day 2								
	Day 3								
	Day 4								
		32.7							
60 (58.9) mg/L	Day 0	58.1	22.0	7.88	8.0	622			
	Day 1	59.6	21.9	7.92	7.5				
	Day 2								
	Day 3								
	Day 4								
		58.9							
100 (89.4) mg/L	Day 0	91.0	22.0	7.89	8.0	781	72	68	ND
	Day 1	87.7	21.9	7.99	7.5		70	68	
	Day 2								
	Day 3								
	Day 4								
		89.4							

^a Fluoride Analysis EPA 300.0
ND Not Detect; below detection limit

Chloride

Table 103 provides a summary of the estimated LC₅₀ value for the toxicity test performed using chloride.

Table 103. LC₅₀ estimate for toxicity test performed using chloride.

Test Species and Duration	LC ₅₀ (mg Cl/L)
<i>Musculium transversum</i> - 96 hr	1,930

For the acute toxicity test completed using chloride, two tables were generated: the first summarizes the test results for the toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity test.

96-hr Toxicity of Chloride on *Musculium transversum*

The 96-hr test to determine the toxicity of chloride on *M. transversum* was completed by INHS. Test organisms, juveniles released from field collected adults, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 8,000, 4,018, 2,028, 1,032, and 535 mg Cl/L.

Testing was conducted at 22 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 104; test results are provided in Table 105. Analytical chemistry data are provided in Table 106. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 31.

Table 104. Test conditions for 96-hour toxicity test on *Musculium transversum* with chloride.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Musculium transversum</i> , juveniles (released from field-collected adults)
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 04 - 08, 2009
4. Test Temperature (°C):	22 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	150 mL beaker
9. Volume of Test Solutions:	120 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg Cl/L):	8000, 4018, 2028, 1032, and 535
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg/L):	7750, 3899, 1900, 985 and 505
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	EPA MHRW
17. Test Material:	Sodium chloride: Sigma Aldrich, 99% pure ACS Reagent Cas. No. 7647-14-5, Lot # B0110043
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 105. Test results for 96-hour toxicity test on *Musculium transversum* with chloride.

Results of a <i>Musculium transversum</i> 96-Hour Static Acute Toxicity Test Conducted 09/04/09 - 09/08/09 Using: Sodium chloride: Sigma Aldrich Cas. No. 7647-14-5								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	5	5	5	2722	2455	2455	1930
535 (505) mg/L	0	0	0	0	96-Hour LC₅₀* = 1930 mg/L			
1032 (985) mg/L	0	0	0	0				
					LC ₅₀ 95% Confidence Limits			
2028 (1900) mg/L	0	15	15	50	24-Hr	48-Hr	72-Hr	96-Hr
4018 (3899) mg/L	100	100	100	100	LL NR	2200	2200	1655
					UL NR	2740	2740	2251
8000 (7750) mg/L	100	100	100	100	LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 106. Analytical chemistry data for 96-hour toxicity test on *Musculium transversum* with chloride.

Nominal (Measured) Test Concentration		Chloride ^a (mg/L)	Sulfate ^b (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (µmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	33	58	23.0	8.1	7.93	385	62	48
	Day 1			22.9					
	Day 2			23.0					
	Day 3			23.0					
	Day 4	39	59	23.0	8.0	8.13	378	62	48
		36							
535 (505) mg/L	Day 0	490	58	22.9	8.1	7.99	2010	62	48
	Day 1			23.0					
	Day 2			22.9					
	Day 3			22.9					
	Day 4	520	59	23.0	8.0	8.14	2000	62	48
		505							
1032 (985) mg/L	Day 0	970	59	22.8	8.1	8.00	3620	62	48
	Day 1			22.9					
	Day 2			22.8					
	Day 3			22.9					
	Day 4	1000	59	23.0	8.0	8.22	3640	62	48
		985							
2028 (1900) mg/L	Day 0	1900	58	22.9	8.0	8.05	6700	62	48
	Day 1			23.0					
	Day 2			22.9					
	Day 3			23.0					
	Day 4	1900	60	23.0	8.0	8.14	6730	62	48
		1900							
4018 (3899) mg/L	Day 0	3800	58	22.9	8.0	8.05	12520	62	48
	Day 1			23.0					
	Day 2			22.7					
	Day 3			22.9					
	Day 4	4000	59	22.9	8.1	8.06	12470	62	48
		3899							
8000 (7750) mg/L	Day 0	7700	60	22.8	8.0	8.07	24200	62	48
	Day 1			22.9					
	Day 2			22.9					
	Day 3			22.8					
	Day 4	7800	60	22.9	7.9	8.14	24000	62	48
		7750							

^a Chloride Analysis Method 300.0

^b Sulfate Analysis Method 300.0

Sulfate

Table 107 provides a summary of estimated LC₅₀ values for the two toxicity tests performed using sulfate. LC₅₀ values ranged between 1,483 and 3,378 mg SO₄/L.

Table 107. LC₅₀ estimates for toxicity tests performed using sulfate.

Test Species and Duration	LC ₅₀ (mg SO ₄ /L)
<i>Ligumia recta</i> - 96 hr	1,483
<i>Megalonaias nervosa</i> - 96 hr	3,378

For each of the acute toxicity tests completed using sulfate, two tables were generated: the first summarizes the test results for each toxicity test, including nominal and analytical test concentration and LC₅₀ estimates with confidence intervals; the second table summarizes analytical chemistry data collected throughout the toxicity tests.

96-hr Toxicity of Sulfate on *Ligumia recta*

The 96-hr test to determine the toxicity of sulfate on *L. recta* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Missouri State University laboratory culture, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 5,000, 2,545, 1,317, 703, and 397 mg SO₄/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 108; test results are provided in Table 109. Analytical chemistry data are provided in Table 110. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 32.

Table 108. Test conditions for 96-hour toxicity test on *Ligumia recta* with sulfate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Ligumia recta</i> , juveniles <5 days old, Missouri State University
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	September 10 - 14, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg SO ₄ /L):	5,000, 2,545, 1,317, 703, and 397
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination-mg SO ₄ /L):	5,038, 2,349, 1,200, 665, and 370
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	USEPA MHRW
17. Test Material:	Sodium sulfate anhydrous: Sigma Aldrich, 99% pure Cas. No. 7757-82-6, Lot # A0231747
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 109. Test results for 96-hour toxicity test on *Ligumia recta* with sulfate.

Results of a <u>Ligumia recta</u>		<u>96-Hour Static Acute Toxicity Test</u>						
Conducted <u>09/10/09 - 09/14/09</u>		<u>Using: Sodium sulfate: Sigma Aldrich Cas. No. 7757-82-6</u>						
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	5	3440	3440	1938	1483
397 (370) mg/L	0	0	0	0	96-Hour LC ₅₀ * = 1483 mg/L			
703 (665) mg/L	0	0	0	5	LC ₅₀ 95% Confidence Limits			
1317 (1200) mg/L	0	0	0	15	24-Hr	48-Hr	72-Hr	96-Hr
2545 (2349) mg/L	0	0	80	100	LL NR	NR	1705	1321
5000 (5038) mg/L	100	100	100	100	UL NR	NR	2203	1665
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

* All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 110. Analytical chemistry data for 96-hour toxicity test on *Ligumia recta* with sulfate.

Nominal (Measured) Test Concentration		Sulfate ^a (mg/L)	Chloride ^b (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	83	3.0	19.8	7.9	7.93	309	60	92
	Day 1			19.7					
	Day 2			19.8					
	Day 3			19.2					
	Day 4	85	2.5	19.2	8.0	7.85	308	62	92
		84							
397 (370) mg/L	Day 0	380	2.6	19.8	8.0	7.95	1029	60	92
	Day 1			19.7					
	Day 2			19.8					
	Day 3			19.2					
	Day 4	360	2.7	19.2	8.2	7.87	1032	62	92
		370							
703 (665) mg/L	Day 0	660	2.8	19.8	8.1	7.95	1691	60	92
	Day 1			19.8					
	Day 2			19.9					
	Day 3			19.2					
	Day 4	670	2.9	19.2	8.3	7.92	1716	60	92
		665							
1317 (1200) mg/L	Day 0	1200	3.9	19.8	8.0	7.94	2840	60	92
	Day 1			19.9					
	Day 2			19.9					
	Day 3			19.2					
	Day 4	1200	3.3	19.2	8.3	7.96	2990	60	92
		1200							
2545 (2349) mg/L	Day 0	2300	4.3	19.8	8.1	7.99	5280	60	92
	Day 1			19.9					
	Day 2			20.0					
	Day 3			19.2					
	Day 4	2400	4.8	19.2	8.4	7.95	5380	60	92
		2349							
5000 (5038) mg/L	Day 0	4700	5.9	19.9	8.1	8.08	9570	62	94
	Day 1			19.9					
	Day 2			20.0					
	Day 3			19.2					
	Day 4	5400	6.1	19.2	8.4	8.00	9680	64	94
		5038							

^a Sulfate Analysis Method 300.0

^b Chloride Analysis Method 300.0

96-hr Toxicity of Sulfate on *Megalonias nervosa*

The 96-hr test to determine the toxicity of sulfate on *M. nervosa* was completed by INHS. Test organisms, < 5-day old juveniles collected from the Genoa National Fish Hatchery, were acclimated to the dilution water (MHRW), test temperature and other test conditions prior to test initiation. Once acclimated, test organisms were examined for any disease, stress, parasites, etc. If free from ailments, test organisms were randomly assigned to the test chambers (which were randomly assigned to testing locations); four replicates were used per treatment with five organisms per replicate.

Organisms were exposed to a dilution water control and the test chemical at varying concentrations under static conditions. Serial dilutions of the highest test concentration (known weight of test chemical dissolved in a known volume of dilution water) were made to prepare the following nominal test concentrations: 5,000, 2,545, 1,317, 703, and 397 mg SO₄/L.

Testing was conducted at 20 ± 1 °C with a photoperiod of 16 hr light and 8 hr dark (ambient laboratory light). Organisms were not fed for the duration of the test and were examined daily for mortality. Once the test was complete, the LC₅₀ value was determined using the Spearman-Kärber method.

A summary of the toxicity test conditions present throughout the assessment are provided in Table 111; test results are provided in Table 112. Analytical chemistry data are provided in Table 113. Accompanying information, including raw laboratory data, analytical chemistry data and statistical analyses, is provided in Appendix 33.

Table 111. Test conditions for 96-hour toxicity test on *Ligumia recta* with sulfate.

Summary of Toxicity Test Conditions	
1. Test Species and Age:	<i>Megaloniaias nervosa</i> , juveniles <5 days old, Genoa National Fish Hatchery
2. Test Type and Duration:	Static, 96 hours
3. Test Dates:	October 16 - 20, 2009
4. Test Temperature (°C):	20 ± 1
5. Light Quality:	Ambient Laboratory, 10-20 µE/m²/s
6. Photoperiod:	16 h light, 8 h darkness
7. Feeding Regime:	None
8. Size of Test Vessel:	50 mL beaker
9. Volume of Test Solutions:	40 mL
10. No. of Test Organisms per Test Vessel:	5
11. No. of Test Vessels per Treatment:	4
12. Total No. of Test Organisms per Treatment:	20
13. Test Concentrations (mg SO ₄ /L):	5,000, 2,545, 1,317, 703, and 397
14. Analytical Test Concentrations (geometric mean of samples collected at test initiation and termination- mg SO ₄ /L):	5,130, 2,498, 1,249, 690, and 380
15. Renewal of Test Solutions:	None
16. Dilution and Primary Control Water:	EPA MHRW
17. Test Material:	Sodium sulfate anhydrous: Sigma Aldrich, 99% pure Cas. No. 7757-82-6, Lot # A0231747
18. Secondary Control Water:	None
19. Aeration:	None
20. Endpoints Measured:	Mortality (LC ₅₀)

Table 112. Test results for 96-hour toxicity test on *Megalonaias nervosa* with sulfate.

Results of a <i>Megalonaias nervosa</i> 96-Hour Static Acute Toxicity Test Conducted 10/16/09 - 10/20/09 Using: Sodium sulfate: Sigma Aldrich Cas. No. 7757-82-6								
Nominal (Measured) Concentrations	Cumulative Percent Affected ^a				LC ₅₀ Values* (mg/L)			
	24-Hr	48-Hr	72-Hr	96-Hr	24-Hr	48-Hr	72-Hr	96-Hr
Primary Control/ Dilution Water	0	0	0	0	3,564	3,564	3,378	3,378
397 (380) mg/L	5	5	10	10	96-Hour LC ₅₀ * = 3,378 mg/L			
703 (690) mg/L	0	0	5	5				
					LC ₅₀ 95% Confidence Limits			
1317 (1249) mg/L	0	0	0	0	24-Hr	48-Hr	72-Hr	96-Hr
2545 (2498) mg/L	0	0	10	10	LL NR	NR	3,021	3,021
					UL NR	NR	3,777	3,777
5000 (5130) mg/L	100	100	100	100				
					LL = Lower Limit UL = Upper Limit NR = Confidence Intervals are not reliable			
					Method(s) Used to Determine LC ₅₀ and EC ₅₀ Confidence Limit Values: Spearman-Kärber			

^a Cumulative percent affected is the total percentage of test organisms observed dead, immobile, exhibiting loss of equilibrium or other defined endpoints.

- All LC₅₀ and EC₅₀ values are determined based on measured concentrations.

Table 113. Analytical chemistry data for 96-hour toxicity test on *Megalonaias nervosa* with sulfate.

Nominal (Measured) Test Concentration		Sulfate ^a (mg/L)	Chloride ^b (mg/L)	Temperature (°C)	pH (s.u.)	D.O. (mg/L)	Conductivity (mmhos)	Alkalinity (mg/L)	Hardness (mg/L)
Dilution water/Control	Day 0	83	2.4	21.0	7.8	7.95	300	60	88
	Day 1			20.9	7.8	8.30	300		
	Day 2			20.5	7.8	8.21	308		
	Day 3			20.9	7.9	8.28	324		
	Day 4	90	2.4	20.6	7.9	8.18	364	68	94
		86	2.4						
397 (380) mg/L	Day 0	380	2.4	21.0	8.0	8.24	1021	60	88
	Day 1			20.8	7.9	8.38	1016		
	Day 2			20.4	7.9	8.22	1020		
	Day 3			20.9	8.0	8.40	1021		
	Day 4	380	2.6	20.7	8.0	8.20	1104	68	88
		380	2.5						
703 (690) mg/L	Day 0	680	2.7	21.0	8.0	8.21	1702	62	90
	Day 1			20.9	8.0	8.35	1695		
	Day 2			20.4	8.0	8.23	1698		
	Day 3			20.9	8.0	8.29	1696		
	Day 4	700	2.8	20.7	8.0	8.25	1853	68	88
		690	2.7						
1317 (1249) mg/L	Day 0	1200	3.1	21.0	8.1	8.28	2940	62	90
	Day 1			21.0	8.0	8.32	2920		
	Day 2			20.5	8.0	8.28	2930		
	Day 3			20.9	8.0	8.40	2940		
	Day 4	1300	3.3	20.8	8.0	8.24	3230	68	90
		1249	3.2						
2545 (2498) mg/L	Day 0	2400	4.0	21.0	8.2	8.22	5320	62	90
	Day 1			20.9	8.1	8.30	5280		
	Day 2			20.5	8.1	8.20	5295		
	Day 3			20.8	8.2	8.38	5290		
	Day 4	2600	4.3	20.8	8.1	8.20	5600	68	92
		2498	4.1						
5000 (5130) mg/L	Day 0	4700	5.7	21.0	8.3	8.26	9550	62	94
	Day 1			21.0	8.2	8.28	9540		
	Day 2			20.5	8.2	8.00	9540		
	Day 3			20.8	8.3	8.32	9540		
	Day 4	5600	6.3	20.9	8.2	8.37	10320	68	92
		5130	6.0						

^a Sulfate Analysis Method 300.0

^b Chloride Analysis Method 300.0

REFERENCES

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF). 2006 a. Standard Methods for the Examination of Water and Wastewater. Method 2340 C (Hardness).

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF). 2006 b. Standard Methods for the Examination of Water and Wastewater. Method 2320 B (Alkalinity).

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF). 2006 c. Standard Methods for the Examination of Water and Wastewater. Method 4500-NH₃ (D).

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF). 2006 d. Standard Methods for the Examination of Water and Wastewater. Method 4500 NO₃ Nitrogen (Nitrate).

American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF). 2006 e. Standard Methods for the Examination of Water and Wastewater. Method 4500 NO₂ Nitrogen (Nitrite).

American Society for Testing and Materials (ASTM). 2005. Standard Guide for Conducting Early Life-Cycle Toxicity Tests with Fishes. Standard E1241-05 in Vol. 11.06 of the Annual Book of ASTM Standards. ASTM International, West Conshohocken, PA.

American Society for Testing and Materials (ASTM). 2006 a. Standard Guide for Conducting Laboratory Toxicity Tests with Freshwater Mussels. Standard E2455-06 in Vol. 11.06 of the Annual Book of ASTM Standards. ASTM International, West Conshohocken, PA.

American Society for Testing and Materials (ASTM). 2006 b. Standard Guide for Conducting Three-Brood, Renewal Toxicity Tests with *Ceriodaphnia dubia*. Standard E1295-01 in Vol. 11.06 of the Annual Book of ASTM Standards. ASTM International, West Conshohocken, PA.

American Society for Testing and Materials (ASTM). 2007. Standard Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians. Standard E729-96 in Vol. 11.06 of the Annual Book of ASTM Standards. ASTM International, West Conshohocken, PA.

Benoit D.A., V.R. Mattson, and D.I. Olson. 1982. A continuous-flow *mini-diluter* system for toxicity testing. Water Res. 16:457-464.

REFERENCES (CONT'D)

Great Lakes Environmental Center (GLEC). 2008. Standard Operating Procedure for the Determination of Nitrate Plus Nitrite Nitrogen in Surface Water Samples Using Automated Hydrazine Reduction. SOP Number: CHM 2008. Effective Date: September 25, 1997. Revision Date: May 5, 2008. 13 pgs.

Great Lakes Environmental Center (GLEC). 2009 a. Standard Operating Procedure for Acute Static Toxicity Tests for Test Materials with *Ceriodaphnia dubia*. SOP Number: TOX 0047. Effective Date: June 2, 2009. Revision Date: June 16, 2009. 6 pgs.

Great Lakes Environmental Center (GLEC). 2009 b. Standard Operating Procedure for Acute Static Toxicity Tests for Test Materials with *Chironomus dilutus*. SOP Number: TOX 0045. Effective Date: May 7, 2009. Revision Date: June 16, 2009. 7 pgs.

Great Lakes Environmental Center (GLEC). 2009 c. Standard Operating Procedure for Acute Static Toxicity Tests for Test Materials with Fathead Minnows. SOP Number: 0044. Effective Date: May 7, 2009. Revision Date: June 19, 2009. 7 pgs.

Great Lakes Environmental Center (GLEC). 2009 d. Standard Operating Procedure for Acute Static Toxicity Tests for Test Materials with *Hyaella azteca*. SOP Number: 0046. Effective Date: May 7, 2009. Revision Date: June 16, 2009. 7 pgs.

Great Lakes Environmental Center (GLEC). 2009 e. Standard Operating Procedure for Early Life-Stage Flow-Through Toxicity Tests with *Pimephales promelas* (Fathead Minnow). SOP Number: TOX 0043. Effective Date: June 2, 2009. Revision Date: June 16, 2009. 10 pgs.

Great Lakes Environmental Center (GLEC). 2009 f. Standard Operating Procedure for Operation of the Continuous Flow-Through Exposure System. SOP Number: TOX 0030. Effective Date: September 1, 1996. Revision Date: June 16, 2009. 4 pgs.

Great Lakes Environmental Center (GLEC). 2009 g. Standard Operating Procedure for Static-Renewal Short-Term Chronic Toxicity Tests for Test Materials with *Ceriodaphnia*. SOP Number: TOX 0042. Effective Date: June 2, 2009. Revision Date: June 16, 2009. 8 pgs.

Great Lakes Environmental Center (GLEC). 2009 h. Standard Operating Procedure for the Colorimetric Determination of Nitrite Nitrogen in Water Samples Using the Milton Roy Spectronic 1200 Spectrophotometer. SOP Number: CHM 2015. Effective Date: April 12, 1999. Revision Date: May 14, 2009. 9 pgs.

Great Lakes Environmental Center (GLEC). 2009 i. Quality Assurance Project Plan: Nitrate, Nitrite, Boron, Manganese, Fluoride and Chloride Toxicity Testing. Prepared for U.S. EPA, Office of Science and Technology, Health and Ecological Criteria Division. EPA Contract #EP-C-09-001, Work Assignment B-12. 33 pgs.

REFERENCES (CONT'D)

Gulley, D.D. 1996. TOXSTAT 3.5. Western Eco Systems Technology, Incorporated. Cheyenne, WY, October 1996.

Illinois Natural History Survey (INHS). 2009. Standard Operating Procedure for Acute Static Toxicity Tests for Test Material with *Sphaerium simile*, *Musculium sp.*, stonefly nymphs, and *Lampsilis siliquoidea*. Effective Date: June 8, 2009. 5 pgs.

Simmerman, R.V. 2008. Standard Operating Procedure for the Analysis of Metals using ICP-MS Technique. Completed for SOS Analytical on October 1, 2008. 8 pgs.

Underwriters Laboratories (UL). 2008 a. Standard Operating Procedure for General Chemistry: Inorganic Anions in Water by Ion Chromatography. SOP Number: UL-SBN-GenChem-008-17. Effective Date: June 17, 2008. 24 pgs.

Underwriters Laboratories (UL). 2008 b. Standard Operating Procedure for Metals: Determination of Trace Elements in Water by Inductively Coupled Plasma-Atomic Emission Spectrometry (ICP-AES). SOP Number: UL-SBN-MET-007-09. Effective Date: November 11, 2008. 29 pgs.

Underwriters Laboratories (UL). 2009 a. Standard Operating Procedure for General Chemistry: Fluoride in Water by Ion Selective Electrode. SOP Number: UL-SBN-GenChem-010-11. Effective Date: March 4, 2009. 17 pgs.

Underwriters Laboratories (UL). 2009 b. Standard Operating Procedure for Metals: Determination of Trace Elements in Water by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS). SOP Number: UL-SBN-MET-004-17. Effective Date: February 19, 2009. 28 pgs.

United States Environmental Protection Agency (EPA). 1971. Method 354.1: Determination of Nitrite by Spectrophotometry.

United States Environmental Protection Agency (EPA). 1978. Method 353.1: Determination of Nitrate-Nitrite by Colorimetry.

United States Environmental Protection Agency (EPA). 1985. Guidelines for Deriving Numerical National Water Quality Criteria for the Protection of Aquatic Organisms and Their Uses. PB85-227049. Washington, DC 20460.

United States Environmental Protection Agency (EPA). 1993. Method 300.0: Determination of Inorganic Anions by Ion Chromatography.

United States Environmental Protection Agency (EPA). 1994. Method 200.7: Determination of Metals and Trace Elements in Water and Wastes by Inductively Coupled Plasma – Atomic Emission Spectrometry.

REFERENCES (CONT'D)

United States Environmental Protection Agency (EPA). 1994. Method 200.8: Determination of Trace Elements in Water and Wastes by Inductively Coupled Plasma – Mass Spectrometry.

United States Environmental Protection Agency (EPA). 2002 a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, Fifth Addition. Office of Water, Washington, DC. EPA-821-R-02-012.

United States Environmental Protection Agency (EPA). 2002 b. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms, Fourth Addition. Office of Water, Washington, DC. EPA-821-R-02-013.